



PHD

Hypoxia in inflammation

Peters, Caren Lorraine

Award date:
2003

Awarding institution:
University of Bath

[Link to publication](#)

Alternative formats

If you require this document in an alternative format, please contact:
openaccess@bath.ac.uk

Copyright of this thesis rests with the author. Access is subject to the above licence, if given. If no licence is specified above, original content in this thesis is licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC-ND 4.0) Licence (<https://creativecommons.org/licenses/by-nc-nd/4.0/>). Any third-party copyright material present remains the property of its respective owner(s) and is licensed under its existing terms.

Take down policy

If you consider content within Bath's Research Portal to be in breach of UK law, please contact: openaccess@bath.ac.uk with the details. Your claim will be investigated and, where appropriate, the item will be removed from public view as soon as possible.

HYPOXIA IN INFLAMMATION

submitted by

Caren Lorraine Peters

For the degree of PhD

University of Bath

2003

The research work in this thesis has been carried out in the Department of Pharmacy and Pharmacology, under the supervision of Dr C. J. Morris.

COPYRIGHT

Attention is drawn to the fact that copyright of this thesis rests with its author. This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognize that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author.

This thesis may be made available for consultation within the University Library and may be photocopied or lent to other libraries for the purposes of consultation.

.....*C. Peters*.....
.....

UMI Number: U488350

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U488350

Published by ProQuest LLC 2014. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

There is strong evidence to support a hypothesis that inflammation in rheumatoid arthritis (RA) may in part be driven by hypoxia resulting from poorly vascularized but highly proliferative tissue. Much work has been carried out that demonstrates hypoxia and hypoxic changes in rheumatoid but not in non-rheumatoid joints.

In this thesis I use the rat adjuvant arthritis model, which mirrors much of the pathology of rheumatoid arthritis to identify hypoxic cells, cytokines and transcription factors involved in joint inflammation. My study demonstrates for the first time the use of Hypoxyprobe-1, a commercially available exogenous probe, to identify hypoxic cells in all structures of the inflamed joint including the synovium, pannus, bone marrow and articular cartilage chondrocytes. It showed that as the clinical symptoms of adjuvant arthritis develop, so the inflamed joint becomes hypoxic and that this hypoxia is significantly higher than naïve controls. Increased cellular hypoxia was associated with a concurrent increase in the expression of Ets-1 transcription factor during the inflammatory response in adjuvant rat joints and in synovial fibroblasts from RA patients. From this I concluded that hypoxia and HIF-1 α may be involved in the upregulation of ets-1 during joint inflammation. My localisation of ets-1 in the invasive pannus is indicative of ets-1 involvement in the invasion of synovial joint cartilage and bone by proliferating synovium.

The results are discussed in light of novel hypoxia targeted therapies and clearly show the suitability of adjuvant arthritis for future in vivo research into the likely efficacy of hypoxia-regulated therapies like bioreductive and hypoxia mediated gene therapy in the treatment of rheumatoid arthritis.

Acknowledgements

I would like to thank my supervisor Dr. Chris Morris for his support, encouragement and enthusiasm throughout this research project.

Hugs to Dr. Viv Winrow and Dr. Tuls Bodamyali for their dedicated proof-reading and an ear for a good whinge.

For the provision of sample tissue and technical advice thanks to Dr. Paul Mapp.

Many thanks to the technicians of 5west level 1 for their assistance.

I would like to thank my postgraduate and postdoctoral colleagues in 7west 2.9 for their friendship, help, advice and regular booze ups that have got me through the years.

I acknowledge financial support from the Arthritis Research Campaign UK.

Finally, I would like to thank my family and fiancé, Rob, for their love and support.

Quotation

“To the doubters I say there is no force greater than self belief.”

Caren Lorraine Peters – 2003.

Table of Contents.

| | |
|---|----|
| HYPOXIA IN INFLAMMATION..... | 1 |
| Abstract | 2 |
| Acknowledgements | 3 |
| Quotation..... | 4 |
| Table of Contents. | 5 |
| List of Figures | 8 |
| List of Plates..... | 9 |
| CHAPTER 1: INTRODUCTION..... | 13 |
| 1.1 The Inflammatory Response..... | 13 |
| 1.2. Rheumatoid Arthritis - An Inflammatory Disease..... | 13 |
| 1.2.1 The Anatomy of the Synovial Joint..... | 14 |
| 1.2.2 The Aetiology of Rheumatoid Arthritis..... | 15 |
| 1.2.3 Genetic Contributions to Rheumatoid Arthritis..... | 16 |
| 1.2.4 Infective Contributions to Rheumatoid Arthritis | 17 |
| 1.2.5 The Histopathology of Rheumatoid Arthritis | 19 |
| 1.3 Hypoxia and Oxygen Measurement | 23 |
| 1.3.1 Direct Methods of Oxygen Measurement..... | 23 |
| 1.3.2 Indirect Methods of Oxygen Detection | 25 |
| 1.4. Evidence of Hypoxia in the Rheumatoid Joint from Human and Animal Studies | 32 |
| 1.5 Mechanisms for Hypoxia in the Joint..... | 33 |
| 1.6 Angiogenesis and Vasculature in the Rheumatoid Joint | 34 |
| 1.7 High Intra-articular Pressure and Hypoxia | 36 |
| 1.8 Ischaemia-Reperfusion Injury Generated Free radicals and Tissue Damage. | 37 |
| 1.9 The Effects of Hypoxia in Rheumatoid Arthritis | 40 |
| 1.9.1 Hypoxia and cytokines | 40 |
| 1.9.2 Hypoxia and Matrix Metalloproteinases (MMPs)..... | 42 |
| 1.9.3 Hypoxia and Other Enzymes..... | 44 |
| 1.9.4 Hypoxia and Gene Control..... | 47 |
| 1.9.5 Hypoxia and Bone Remodelling..... | 49 |
| 1.9.6 Concluding remarks. | 50 |
| 1.10 Treatment of Rheumatoid Arthritis | 51 |
| 1.10.1 Analgesics | 52 |
| 1.10.2 Non-steroidal Anti-inflammatory Drugs (NSAIDs)..... | 52 |
| 1.10.3 Corticosteroids..... | 53 |
| 1.10.4 Disease Modifying Anti-rheumatic Drugs (DMARDs)..... | 54 |
| 1.10.5 Therapies from research and development: Targeting hypoxia..... | 56 |
| 1.10.6 Therapies from research and development: Anti Cytokine therapy | 58 |
| 1.10.7 Therapies from research and development: Angiogenesis Inhibitors..... | 59 |
| 1.10.8 Therapies from research and development: Gene Therapy | 61 |
| 1.10.9 Therapies from research and development: The final comment..... | 64 |
| 1.11. Animal models in the study of rheumatoid arthritis | 64 |
| 1.11.1 Detection of hypoxia in the adjuvant arthritis model | 65 |
| 1.11.2 Other animal models..... | 67 |
| 1.12 Study Hypothesis..... | 70 |
| CHAPTER 2: MATERIALS AND METHODS..... | 71 |
| 2.1. Materials and methods for animal model | 71 |
| 2.1.1. Animals | 71 |
| 2.1.2. Materials for induction of Adjuvant Arthritis in the animal model | 71 |
| 2.1.3 Materials for labelling of hypoxic cells in animal models using Hypoxyprobe | 71 |
| 2.1.4 Animal husbandry | 72 |
| 2.1.5 Induction of Adjuvant Arthritis | 72 |
| 2.1.6 Clinical assessment..... | 73 |
| 2.1.7 Labelling of hypoxic cells using Hypoxyprobe..... | 73 |
| 2.1.8 Termination of the animal model | 73 |

| | |
|---|-----|
| 2.2 Materials and methods for histological studies | 74 |
| 2.2.1 Materials for tissue processing | 74 |
| 2.2.2 X-ray materials | 74 |
| 2.2.3 Materials for histological assessment | 75 |
| 2.2.4 Antibodies for immunohistochemical staining | 76 |
| 2.2.5 Tissue processing methods, paraffin embedding | 77 |
| 2.2.6 Tissue processing methods, snap freezing | 77 |
| 2.2.7 X-ray of joints | 77 |
| 2.2.8 Haematoxylin and Eosin staining of sections | 78 |
| 2.2.9 Immunohistochemical staining methods | 79 |
| 2.2.10 Histology imaging | 83 |
| 2.2.11 Histological assessment | 83 |
| 2.2.12 Immunohistochemical assessment | 84 |
| 2.3 Statistics | 85 |
| 2.4 Materials and methods for tissue explant and cell culture | 85 |
| 3.4.1 Cell and tissue culture materials | 85 |
| 2.4.2 Cell lines and tissue explants | 86 |
| 2.4.3 Defrosting cells and culturing cells | 86 |
| 2.4.4 Passaging cells | 87 |
| 2.4.5 Freezing down cells | 87 |
| 2.4.6 Culturing tissue explants | 88 |
| 2.5 Materials and method for hypoxia cabinet operation | 88 |
| 2.5.1 Materials for hypoxia cabinet operation | 88 |
| 2.5.2 Method for hypoxia cabinet operation | 89 |
| 2.6 Materials and methods for protein isolation | 91 |
| 2.6.1 Materials for protein isolation | 91 |
| 2.6.2 Method for protein isolation | 91 |
| 2.7 Materials and Methods for Protein Assay | 92 |
| 2.7.1 Materials for Protein Assay | 92 |
| 2.7.2 Method for Protein Assay | 93 |
| 2.8 Materials and methods for gel electrophoresis & western blotting | 93 |
| 2.8.1 Materials for Gel Electrophoresis & Western Blotting | 93 |
| 2.8.2 Preparation of the polyacrylamide gel plates | 94 |
| 2.8.3 Sample solution Preparation | 95 |
| 2.8.4 Gel Electrophoresis | 95 |
| 2.8.5 Western Blot and Immunodetection by ECL | 95 |
| 2.8.6 Assessment of western blots | 98 |
| CHAPTER 3: Determining the Optimum Time Point for Administering the Hypoxyprom | 99 |
| 3.1 Introduction | 99 |
| 3.2 Materials and Methods | 99 |
| 3.3 Results | 101 |
| 3.4 Discussion | 106 |
| CHAPTER 4: Characterisation of the Time Course of Hypoxia in the Synovial Joint of Adjuvant Arthritis | 107 |
| 4.1 Introduction | 107 |
| 4.2 Materials and Methods | 107 |
| 4.3 Results | 108 |
| 4.3.1 Results of Histological Assessment | 110 |
| 4.3.2 Results of Immunohistochemical assessment | 116 |
| 4.4 Discussion | 129 |
| 4.5 Conclusions | 132 |
| CHAPTER 5: Hypoxia, Cell Characterisation, Transcription factors and Cytokines | 133 |
| 5.1 Introduction | 133 |
| 5.2 Materials and Methods | 135 |
| 5.3 Results | 136 |
| 5.3.1 Results of Histological Assessment | 136 |
| 5.3.2 Results of Immunohistochemical Assessment | 137 |

| | |
|--|-----|
| 5.4 Discussion | 154 |
| CHAPTER 6: Effect of Induced Hypoxia on Transcription Factors <i>Invitro</i> | 159 |
| 6.1 Introduction | 159 |
| 6.2 Materials and Methods | 160 |
| 6.3 Results | 161 |
| 6.3.1 Effect of hypoxia on ets-1 and HIF-1 α protein expression in synovial fibroblasts from rheumatoid patients (RSF)..... | 161 |
| 6.3.2 Effect of hypoxia on ets-1 and HIF-1 α protein expression in rat tissue explants..... | 163 |
| 6.3.3 Histological assessment of rat tissue explants from normoxic and hypoxic culture..... | 169 |
| 6.4 Discussion | 174 |
| CHAPTER 7: Summary of Conclusions and General Discussion..... | 178 |
| CHAPTER 8: Future Work | 184 |
| References | 186 |
| Appendix A | 226 |
| Appendix B | 229 |

List of Figures

| | |
|---|-----|
| Figure 1. Diagram of a normal synovial joint | 14 |
| Figure 2. Representative diagram of the top view of a hypoxia cabinet (Microflow Anaerobic System)..... | 89 |
| Figure 3. Western blotting tank setup..... | 97 |
| Figure 4. Changes in body weight during the development of adjuvant arthritis. | 109 |
| Figure 5. Development of clinical symptoms over time of adjuvant arthritis rats..... | 109 |
| Figure 6. Change in the histological scores with development of adjuvant arthritis. | 111 |
| Figure 7. Day 3 post adjuvant arthritis induction. Hypoxic area of adjuvant rats (n=5) compared to naïve controls (n=5). P>0.05 (Mann Whitney test.)..... | 116 |
| Figure 8. Day 7 post adjuvant arthritis induction. Hypoxic area of adjuvant rats (n=5) compared to naïve controls (n=5). P>0.05 (Mann Whitney test.)..... | 117 |
| Figure 9. Hypoxic area of adjuvant rats with clinical score 1 (n=7) compared to time matched naïve controls (n=7). P<0.001 (Mann Whitney test.) | 117 |
| Figure 10. Hypoxic area of adjuvant rats with clinical score 2 (n=7) compared to time matched naïve controls (n=7). P<0.001 (Mann Whitney test.) | 118 |
| Figure 11. Hypoxic areas of adjuvant rats with clinical score 3 (n=9) compared to time matched naïve controls (n=9). P<0.001 (Mann Whitney test.)..... | 118 |
| Figure 12. Summary of immunohistochemical results in fresh frozen tissue. | 137 |
| Figure 13. Proportion of nuclei with positive Ets- immunoreactivity. | 152 |
| Figure 14. Proportion of hypoxic tissue. | 153 |
| Figure 15. Proportion of nuclei HIF1 α -ir colocalizing with ets-1- ir. | 153 |
| Figure 16a. Western Blot: Effect of hypoxia on HIF-1 α protein expression. | 162 |
| Figure 17a. Western Blot: Effect of hypoxia on ets-1 protein expression. | 163 |
| Figure 18. Effect of hypoxia on ets-1 protein expression by adjuvant and naïve rat tissue explants. | 164 |
| Figure 19a. Western Blot: Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants. | 165 |
| Figure 20a. Western Blot: Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants. | 166 |
| Figure 21a. Western Blot: Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants. | 167 |
| Figure 22a. Western Blot: Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants. | 168 |

List of Plates

| Plate Number | | Page No. |
|-------------------------|---|-----------------|
| 3.1 | Hypoxyprobe negative control, without hypoxyprobe. | 102 |
| 3.2 | Hypoxyprobe negative control, without primary antibody. | 102 |
| 3.3 | Hypoxyprobe negative control, with irrelevant primary antibody. | 102 |
| 3.4 | 30 min hypoxyprobe staining in the adjuvant hind foot. | 103 |
| 3.5 | 2 hour hypoxyprobe staining in the adjuvant hind foot. | 103 |
| 3.6 | 6 hour hypoxyprobe staining in the adjuvant hind foot. | 104 |
| 3.7 | 18 hour hypoxyprobe staining in the adjuvant hind foot. | 104 |
| 3.8 | 6 hour hypoxyprobe staining in the naive hind foot. | 105 |
| 4.1 | Day 3 H&E staining of the hind foot of adjuvant arthritis rat. | 112 |
| 4.2 | Day 3 H&E staining of the hind foot of naive rat. | 112 |
| 4.3 | Day 7 H&E staining of the hind foot of adjuvant arthritis rat. | 113 |
| 4.4 | H&E staining of the hind foot of adjuvant arthritis rat with clinical score 1. | 113 |
| 4.5 | H&E staining of the hind foot of adjuvant arthritis rat with clinical score 2. | 114 |
| 4.6 | H&E staining of the hind foot of adjuvant arthritis rat with clinical score 3. | 114 |
| 4.7 | H&E staining of the hind foot of adjuvant arthritis rat with clinical score 4. | 115 |
| 4.8 | Hypoxyprobe staining in day 0 adjuvant hind foot. | 121 |
| 4.9 | Hypoxyprobe staining in day 0 naive hind foot. | 121 |
| 4.10 | Hypoxyprobe staining in day 3 adjuvant hind foot. | 122 |
| 4.11 | Hypoxyprobe staining in day 3 naive hind foot. | 122 |
| 4.12 | Hypoxyprobe staining in day 7 adjuvant hind foot. | 123 |
| 4.13 | Hypoxyprobe staining in day 7 naive hind foot. | 123 |

| Plate Number | | Page No. |
|-------------------------|---|-----------------|
| 4.14 | Hypoxyprobe staining in adjuvant hind foot with clinical score 1. | 124 |
| 4.15 | Hypoxyprobe staining in adjuvant hind foot with clinical score 1. | 124 |
| 4.16 | Hypoxyprobe staining in time matched (C1) naive hind foot. | 125 |
| 4.17 | Hypoxyprobe staining in adjuvant hind foot with clinical score 2. | 125 |
| 4.18 | Hypoxyprobe staining in time matched (C2) naive hind foot. | 126 |
| 4.19 | Hypoxyprobe staining in clinical score 2 hind foot joint chondrocytes. | 126 |
| 4.20 | Hypoxyprobe staining in clinical score 3 hind foot joint chondrocytes. | 127 |
| 4.21 | Time matched (C3) naive hind foot chondrocytes. | 127 |
| 4.22 | Hypoxyprobe staining in adjuvant hind foot with clinical score 3. | 128 |
| 4.23 | Hypoxyprobe staining in adjuvant hind foot with clinical score 3 | 128 |
| 4.24 | Hypoxyprobe staining in time matched (C3) naive hind foot. | 128 |
| 5.1 | Negative control: Adjuvant hind foot joint, stained using irrelevant mouse antibody. | 139 |
| 5.2 | Negative control: Adjuvant hind foot joint, stained using goat IgG. | 139 |
| 5.3 | Negative control: Adjuvant hind foot joint, stained using rabbit IgG. | 139 |
| 5.4 | Naive hind foot stained with mouse anti-rat ED1. | 140 |
| 5.5 | Naive hind foot stained with mouse anti-rat ED2. | 140 |
| 5.6 | Adjuvant hind foot stained with mouse anti-rat ED1. | 142 |
| 5.7 | Adjuvant hind foot stained with mouse anti-rat ED1. | 142 |
| 5.8 | Naive hind foot joint stained with rabbit anti-iNOS. | 143 |
| 5.9 | Adjuvant hind foot joint stained with rabbit anti-iNOS. | 143 |
| 5.10 | Naive hind foot joint stained with goat anti-VEGF. | 144 |

| Plate Number | | Page No. |
|-------------------------|---|-----------------|
| 5.11 | Adjuvant hind foot joint stained with goat anti-VEGF. | 144 |
| 5.12 | Adjuvant hind foot joint stained with rabbit anti-ets1. Co-localizing with hypoxypromide adducts in plate 6.13. | 146 |
| 5.13 | Adjuvant hind foot joint stained with mouse anti-Hypoxypromide. | 146 |
| 5.14 | Adjuvant hind foot joint stained with rabbit anti-ets1. Co- localizing with hypoxypromide adducts in plate 6.15. | 147 |
| 5.15 | Adjuvant hind foot joint stained with mouse anti-Hypoxypromide. | 147 |
| 5.16 | Naive hind foot joint x40, wax section, stained with rabbit anti- ets1 (insert x10 of same section). Low level, staining for ets-1. | 148 |
| 5.17 | Naive hind foot joint x40, wax section, stained with mouse anti- hypoxypromide (insert x10 of same section). Low level, non nuclei staining, no co-localization with plate 6.16 | 148 |
| 5.18 | Adjuvant hind foot joint stained with rabbit anti-ets1. Co- localizing with HIF-1 α in plate 6.19. | 149 |
| 5.19 | Adjuvant hind foot joint stained with mouse anti-HIF-1 α . | 149 |
| 5.20 | Adjuvant hind foot joint stained with rabbit anti-ets1. Co- localizing with HIF-1 α in plate 6.21 | 150 |
| 5.21 | Adjuvant hind foot joint stained with mouse anti-HIF-1. Co- localizing with ets-1 α in plate 6.20. | 150 |
| 5.22 | Naive hind foot joint x40, wax section, stained with rabbit anti- ets1 (insert x10 of same section). Low level, staining for ets-1. | 151 |
| 5.23 | Naive hind foot joint x40, wax section, stained with mouse anti- HIF-1 α (insert x10 of same section). Low level, no nuclear staining for HIF-1 α . | 151 |
| 6.1 | Adjuvant knee synovium (x5, wax section, haematoxylin and eosin) with a high clinical score (3). Not cultured. | 170 |

| Plate Number | | Page No. |
|-------------------------|--|-----------------|
| 6.2 | Adjuvant knee synovium (x5, wax section, haematoxylin and eosin) with a high clinical score (3). Cultured in 0% O ₂ . | 170 |
| 6.3 | Adjuvant knee synovium (x5, wax section, haematoxylin and eosin) with a high clinical score (3). Cultured in 20%O ₂ . | 171 |
| 6.4 | Naive knee synovium (x5, wax section, haematoxylin and eosin) with zero clinical score. Not cultured. | 171 |
| 6.5 | Naive knee synovium (x5, wax section, haematoxylin and eosin) with a zero clinical score. Cultured in 0% O ₂ . | 172 |
| 6.6 | Naive knee synovium (x5, wax section, haematoxylin and eosin) with a zero clinical score. Cultured in 20%O ₂ . | 172 |

CHAPTER 1: INTRODUCTION

1.1 The Inflammatory Response

The inflammatory response is the process by which both the cellular and molecular components of the immune system are concentrated at a site of infection or tissue damage (Reeves et al., 1991). Essentially it is a protective response by the body to confine damage and assist in the repair of tissues, as well as preventing further invasion of the tissue by foreign organisms. The inflammatory response is normally highly regulated but in some pathological conditions it may become deregulated leading to damage of healthy tissue.

1.2. Rheumatoid Arthritis - An Inflammatory Disease.

Chronic inflammatory disease is a major cause of morbidity and mortality and is well exemplified by rheumatoid arthritis (RA).

This disease is characterized by polyarthritis of synovial joints but because of its unclear aetiology it has been difficult to classify cases. However, the worldwide distribution is widely quoted as about 1-2% of the population according to the American Rheumatism Association criteria (Lawrence et al 1989). Disease onset is usually in the middle years of life, with a female to male ratio of 3:1. There is also a crippling form of this disease that affects juveniles. RA patients suffer considerable morbidity resulting from joint pain, swelling, deformity and the resulting disability. The term rheumatoid arthritis

was first used in 1858 by Sir Archibald Garrod and the pathological changes associated with the inflammatory response have been extensively described since.

1.2.1 The Anatomy of the Synovial Joint

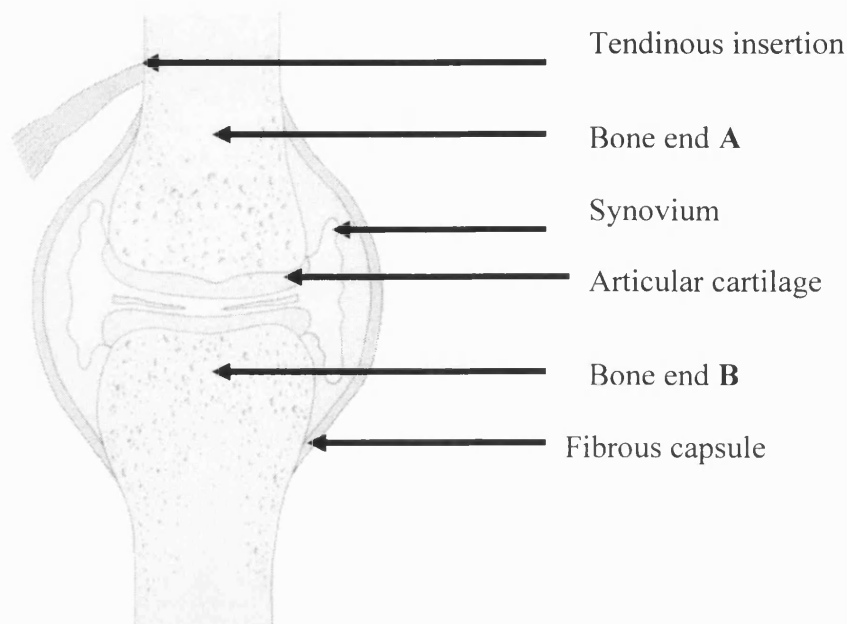


Figure 1. Diagram of a normal synovial joint

The above diagram (Figure 1) shows the complex structure of a synovial joint which is the principle site of inflammation in rheumatoid arthritis. The anatomy of the synovial joint allows two bone ends (**A&B**) to angle, slide and twist. The two bone ends are covered in hyaline cartilage surrounded by a fibrous capsule and ligaments, which enables the bone surfaces to be maintained in apposition. A specialized connective tissue layer called the synovium lines the inner surface of this capsule. Folding of synovial tissue forms villi which enable the joint to change shape during movement. The cells of the synovium can secrete and drain synovial fluid, which has hydraulic and lubricant properties

(Edwards, 1989). The synovial lining is partly in direct contact with the cartilage surface and brings vascular supply to the chondrocytes enabling the provision of nutrients and the removal of waste products.

The intimal lining of the synovium has one side interacting with matrix fibres of the synovium and one side towards the synovial fluid. It does not have a basement membrane or the tight junctions of an epithelium. Two types of synovial cells have been identified in the normal two to three cell thick synovium lining. They were originally classed 'Type A' and 'Type B' cells according to their ultrastructure (Barland et al., 1962) but are now more often referred to by their cell lineage, macrophage-like cells and fibroblast-like cells respectively (Firestein, 2003).

It has been suggested by Barland et al (1962) that the fibroblast-like cells originated from mesenchymal stem cells possessing secretory features and an active Golgi apparatus. Edwards et al (1982) have evidenced histological studies to show that it is the fibroblast-like cells and not the macrophage-like cells that are responsible for joint cavity formation.

The macrophage-like cells have phagocytic capabilities and are responsible for the elimination of microorganisms that may enter the joint via the blood supply and debris from articular wear and tear. As will be described later these cells play an important role when the joint becomes inflamed.

1.2.2 The Aetiology of Rheumatoid Arthritis

As previously mentioned the cause of rheumatoid arthritis (RA) is unknown. The disease shows a large inter-patient variation and there are few animal models that accurately reflect the human disease to enable the study of causative factors. However,

studies of the disease pathology have enabled identification of some contributory factors, which fall into two broad categories.

i) Genetic.

ii) Infective.

1.2.3 Genetic Contributions to Rheumatoid Arthritis

Epidemiologists studying complex diseases use familial clusters and concordance in dizygotic and monozygotic twins to study disease prevalence. From these studies it was established that genetic factors account for approximately sixty percent risk for developing RA (Lawrence, 1970; MacGregor et al., 2000; Maini et al., 1995; Silman et al., 1993). The most extensively studied association was with polymorphic human leukocyte antigen (HLA) alleles within the major histocompatibility complex (MHC) and susceptibility to RA, particularly HLA-DR1 and HLA-DR4.

The particular nucleotide sequence for the gene encoding the constituent molecules of HLA-DR suggest that the structure associated with RA susceptibility resides on the cell surface adjacent to the T-cell receptor molecule (Sewell and Trentham, 1993; Panayi, 1995). This combined with the presence of T cells in RA inflammatory infiltrate (Firestein and Zvaifler, 1990) indicated a crucial role for T-cells in the pathogenesis of RA. This was supported by animal experiments that showed that T-cell clones from adjuvant arthritis transferred disease to naïve recipients (Cohen et al, 1985a).

Levels of rheumatoid factor (RF), auto antibodies specific for immunoglobulin G, correlate with disease activity and suggest a role for auto-immunity in the pathogenesis of RA; approximately 80% of patients are RF seropositive (Firestein, 2003). However, it has

not been proven that RA is an autoimmune disease. Erosion of cartilage and bone can occur independent of T-cell regulation (Muller-Ladner et al, 1995). Also Anti-T cell therapies in RA patients have had little beneficial affect (Fox, 1995). The lack of cures from therapies designed to target specific areas of the immune system have lead to the idea that the out of control immune response is not the primary cause of disease.

1.2.4 Infective Contributions to Rheumatoid Arthritis

It has been suggested that an infectious agent may be the etiological trigger. One theory is that infection by agents that mimic host molecular structures cause autoimmune responses through the breakdown in tolerance of self (Firestein et al, 2000). The concept that an infection could contribute to the etiology rheumatoid arthritis (RA) has been around for many years and in some cases there is a definite link i.e. removal of the organism (or toxic products) by tissue or specific therapy can cause remission of joint symptoms. This is the case in Lyme Disease caused by infection with the spirochete *Borrelia burgdorferi* which leads to inflammation in the joints (Burmester et al., 1995). This is also true for the reactive arthritis induced by either *Chlaymidia trachomatis*, *Salmonella*, *Yersinia*, *Shigella* or *Campylobacter* (Sieper et al., 1996.) Antigens for bacteria such as *Streptococcus pyogenes* and *Mycobacterium tuberculosis* have been found in the synovial fluid of individuals suffering from RA (Klasen et al., 1993) but the significance of this is not known. The fact that arthritis can be induced in animals by injected bacterial preparations supports these studies. For example, rat adjuvant poly-arthritis is induced by *Mycobacterium tuberculosis* (Harris, 1990) and it has been shown that a mycobacterial protein demonstrates immunological cross-reactivity to a cartilage link protein (van Eden et

al., 1985). Even with PCR pan bacterial screens showing bacterial DNA in synovial fluid of RA patients, it is still unclear whether the presence of these organisms is specifically related to the disease process (Gerard et al, 2001).

Evidence showing that some viruses cause polyarthritis similar to RA has been used to support the case for their involvement in the etiology of RA. Rubella can cause short term polyarthritis (Mitchell et al, 1993). Parvovirus B19 causes self-limiting non-destructive arthritis in infected adults and progression to erosive RA has been reported occasionally (Harris, 1990; Gendi et al., 1996). The retrovirus, HTLV-1 can induce polyarthritis in humans and its similarity to RA has lead to the development of a transgenic mouse model, which should help in the study of the role of retroviruses in pathogenesis (Iwakura et al, 1991).

Epstein - Barr virus (EBV) has been linked to RA, mainly due to the large titers of antibody to EBV antigens found in patients (Venables et al., 1988; Harris, 1990; Maini et al., 1995). A shared epitope has also been found between EBV and HLA-DR1 (Roudier et al., 1989), which could increase the susceptibility of infection with the virus. Research in this line seems set to continue as more recently Balandraud (2003) and co-workers have used modern techniques of PCR to demonstrate an increase in EBV in RA patients compared to normal controls.

No one infectious agent has been found to trigger classical rheumatoid arthritis and it is clear that many different factors could over time play a key role in both the pathogenesis and prognosis of RA.

1.2.5 The Histopathology of Rheumatoid Arthritis

There is a large variation in the histopathology both between patients and even within a single joint. However a common feature is cellular hypertrophy and hyperplasia within the synovium (Schumacher and Kitridou, 1972; Poulter et al., 1982; Cush and Lipsky, 1991; Fitzgerald and Bresnihan, 1995; Tak et al., 1997). In the early stages of RA there is an infiltration of polymorphonuclear leukocytes but as the disease progresses and the inflammation becomes more chronic, lymphocytes and mononuclear cells predominate in the infiltrate (Cush and Lipsky, 1991). The synovium also contains lymphoblasts and plasma cells that migrate into the synovium and differentiate into immunoglobulin (Ig) - and rheumatoid factor (RF) - secreting plasma cells (Kobayashi and Ziff, 1973). There is evidence that supports a key characteristic of the rheumatoid joint, the rapid local division of fibroblast-like cells in the synovial lining (Ghadially, 1983). This includes the expression of some proliferation-associated genes *c-myc*, shown to be elevated in synovial tissues from patients with early destructive RA (Trabandt et al., 1992a, b).

Hyperplasia of the rheumatoid synovium may be partly explained by the action of cytokines. Basic fibroblastic growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGF- β), granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin 1 β (IL- β) have been shown to stimulate DNA synthesis and proliferation of human synovial fibroblast-like cells in culture (Butler et al., 1989; Bucala et al., 1991; Goddard et al., 1992). Production of platelet-derived growth factor B chain (PDGF-B/c-sis) and heparin binding acidic fibroblast growth factor-1 has been demonstrated in the rheumatoid synovium (Remmers et al., 1991). Additional to the actions of cytokines, the increase in fibroblast-like cells is also thought to be related to

impaired apoptosis. Matsumoto et al., (1996) observed that apoptotic cells were mainly located in the deep sublining layers of the rheumatoid and that synovial fibroblasts located in the lining layer were not apoptotic and some expressed the anti-apoptosis gene product Bcl-2. They suggested that this would extend the life of the fibroblasts invading the cartilage. Several other anti-apoptotic molecules have been identified including downstream modulators of Fas-signaling, such as sentrin-1 (Franz et al., 2000), small ubiquitin-like modifier (SUMO)-1 and Fas-associated death domain-like interleukin (IL)-1beta-converting enzyme-inhibitory protein (FLIP), as well as transcriptional regulators such as nuclear factor kappa B (NFκB), p53 tumour suppressor gene (p53) (Baier et al., 2003).

As the disease progresses the hyperplastic synovium develops villous projections that protrude into the joint cavity. The tissue at the synovium-cartilage junction is described as fibrous and referred to as “pannus”. It is associated with erosion of the underlying cartilage and bone. Mitrovic (1985) suggested that chondrocytes contribute to the formation of the pannus. Chondrocytes are activated by inflammatory cytokines, dedifferentiate, enlarge their lacunae, coalesce and finally break onto the cartilage surface to form a fibrous pannus. It has also been suggested that “pannus” forms after the collapse of aggressive cell formation and is a relic of the aggressive phase (Fassbender, 1998).

Cells in rheumatoid synovial tissue also contain rhomboid cells that are frequently described as having a ‘transformed’ phenotype. They respond to stimulation by cytokines and growth factors produced in the local inflammatory milieu but also their cellular changes are maintained in the absence of external stimulation (Pap et al 2000).

To date no single specific marker for this phenotype has been identified, instead a combination of characteristics has been observed which lead to the cells aggressive, invasive behavior. In 1989 Lafyatis et al observed the transformed phenotype of synoviocytes from arthritic joints and demonstrated that they were capable of anchorage-independent growth. In 1997 Zvailfler et al gave these cells the name pannocytes to distinguish them from fibroblast like synoviocytes. They have been described as having a rhomboid morphology, hypochromic nuclei with prominent nucleoli and they could also be maintained in culture for longer than fibroblast like synoviocytes (Zvalier et al, 1997). Work has also been carried out to elucidate the genotype of these cells. They have been shown to express mRNA for type I collagen, strong expression of vascular cell adhesion molecule-1 (VCAM-1) (Zvalier et al, 1997; Xue et al, 1997) and the cytokines, tumour necrosis factor alpha (TNF- α), interleukin-1 (IL-1), stromal cell-derived factor 1 (SDF-1) (Seki et al., 1998). Other characteristics include the expression of members of the activator protein-1 (AP-1) transcription factor family, *c-jun* and *c-fos* (Roivainen et al, 1996), which regulate the transcription of pro-inflammatory molecules IL-1, IL-6, and metalloproteinases (Angel et al., 1991, Dooley et al, 1996). In 1992 Trabandt et al (199b) detected c-fos and fibroblast-type collagenase within synovial fibroblast-like cells attached to bone erosions. Tsuji et al (2000) have identified a role for c-fos in cartilage destruction in rheumatoid arthritis. Shiozawa et al (1992) showed that *c-fos* transgenic mice develop an antigen-induced arthritis that occurs independently of lymphocytic infiltration. When the lesions were examined ultra structurally, the majority of the cells invading the cartilage and bone in the destroyed joints were found to possess a 'transformed' appearance distinct from the macrophage-like and fibroblast-like synoviocytes.

Other proteins have been identified in proliferating RA synovial cells including members of the Ras and Raf family of proteins (Muller-Lander et al, 1995). Ras proteins are GTPases involved in signalling pathways between cell surface receptors nuclei or cytoskeletal components. They can bind Raf proteins which can in turn activate the mitogen-activated protein kinase (MAPK) signaling pathways responsible for cell proliferation (Kitasato et al., 2001). In 1991 Trabandt and co workers reported that immunolocalization of Ras and Myc proteins was found in about 70% of the RA cases and was restricted to the proliferating synovial lining cells. Trabandt et al. also demonstrated the enhanced transcription of proteolytic enzymes, like cathepsin B in synovial cells when compared with normal fibroblasts and cathepsin L, a ras-induced protein, was detected in 50% of the RA cases, predominantly in synovial cells attached to cartilage and bone at the site of joint destruction (Trabandt et al., 1990 and 1991).

To be discussed in more detail later in 1.6, vascular changes have also been observed in the rheumatoid synovium; these include vascular congestion and oedema (Rothschild and Masi 1982). Stevens et al (1991a) observed that the capillary density in RA biopsies was reduced, and with greater spatial distribution compared to normal synovium. Also the average distance of the capillaries from the joint cavity was increased. These vascular changes suggest that the rheumatoid joint may be subject to reduced oxygen supply leading to tissue hypoxia and indeed, as will be discussed further in 1.4 and 1.5, there is much evidence to support this. The next section 1.3 will first seek to define hypoxia and discuss the various techniques available for measuring oxygen tension and demonstrate hypoxia in the inflamed joint.

1.3 Hypoxia and Oxygen Measurement

If we consider the various oxygen tensions found in the body it is observed that arterial oxygen tension (pO_2) is 80-90 mmHg and on the venous side it is 36-46 mmHg (Schaf et al., 1988). Low oxygen tensions are often associated with disease, for example *in vivo* models of tumours the oxygen tension is typically 1-9 mmHg (Adam et al., 1999). In this situation the tissue is described as hypoxic. However, before reviewing the evidence for hypoxia in the rheumatoid joint this next section will deal briefly with the methods by which oxygen can be measured and therefore hypoxia detected.

Methods for oxygen measurement can be classified into two types:

1. Direct methods of oxygen measurement.
2. Indirect methods of oxygen measurement.

1.3.1 Direct Methods of Oxygen Measurement

1.3.1.1 Oxygen Probes

Oxygen probes are made from non metallic materials with a solid surface to which a voltage is applied. Oxygen is reduced by this voltage and generates a current, which can be measured and related to concentration of oxygen. These probes are sensitive to a wide range of physiological oxygen tensions. An example of a commercial employment of this technique is the Eppendorf Histogram. However, this method does have some disadvantages; the probes are invasive and therefore cause tissue damage which may in itself lead to inaccurate measurements. The electrodes may sample oxygen from both oxygenated and hypoxic areas at the same time, therefore specific hypoxic cells cannot be

identified. Another disadvantage is the probe may consume some oxygen during measurement thus altering the oxygen concentration preventing accurate repeat measurements in the same area (Swartz, 2002). Blake et al (1997) used an Eppendorf oxygen probe intra-articularly to measure the partial pressure of oxygen in the knees of rheumatoid and non- rheumatoid patients. They showed that only rheumatoid joints had a majority of pO_2 measurements in the very low range of 0-15mmHg.

1.3.1.2 Electron Spin Resonance Oximetry

This technique has utilises the ability of paramagnetic materials, which have unpaired electrons, to interact with oxygen in a specimen. Examples of commonly used paramagnetic materials include fusinite and lithium phthalocyanine. They are implanted into the tissue and the spectral line width as a function of the local pO_2 can be measured non-invasively, frequently and repeatedly from the surface (Swartz, 2002). This technique has undergone considerable development by Swartz and co workers and has been suggested as a suitable technique for clinical measurements in tumours or vascular disease on account of its accuracy, low toxicity and low invasiveness (Swartz and Walczak, 1998). However, most recently it has mainly been used in small animal studies measuring oxygen tension in the brain (Rolett et al., 2000, Lei et al., 2001, Hou et al 2003). Bruce (1992) explored the feasibility of using the technique to measure oxygen tensions in inflamed joints in rats using fusinite and lithium phthalocyanine. However, I can find no further literature suggesting its use in studies of rheumatoid arthritis.

1.3.2 Indirect Methods of Oxygen Detection

1.3.2.1 Nuclear Magnetic Resonance and Magnetic resonance imaging

Nuclear magnetic resonance (NMR) is a form of absorption spectroscopy and may be used *in vivo* studies. The specimen of interest is exposed to electro-magnetic radiation. The specimen nuclei absorb the energy from the electromagnetic radiation; they become excited and are promoted to a higher energy state. As the nuclei return to their original energy state they release an emission which can be measured. This emission varies dependent on the chemical species present. A variety of parameters can be measured from this information including metabolite concentration and pH. NMR can also be used to directly measure P_{O_2} by measuring the oxygen sensitive T_1 relaxation rate of perfluorocarbons injected into the tissue of interest its sensitivity is within ± 2 mmHg (Swartz, 2002). Nuclear magnetic resonance analysis was used to demonstrate increased levels of lactate and ketone bodies such as 3-D-hydroxybutyrate and acetoacetate, as well as decreased concentrations of glucose, chylomicron- and very-low-density lipoproteins (VLDL)-associated triacylglycerols in RA synovial fluid and sera (Naughton et al., 1993a,b).

1.3.2.2 Endogenous Markers of Hypoxia.

The previously described methods will give a global measurement of oxygen *in vitro* and *in vivo*. However, they cannot identify hypoxia at a cellular level. It is known that cells in hypoxic conditions undergo a host of molecular changes that can be detected using

immunohistochemistry. Suggested markers come from the oxygen regulated stress proteins.

Hypoxia activates a basic helix-loop PAS transcription factor; hypoxia inducible factor 1 (HIF-1) (Wenger and Gassman, 1997) HIF-1 is a heterodimer composed of HIF-1 α and HIF-1 β subunits. The HIF-1 α subunit is oxygen regulated and determines HIF-1 activity (Jiang et al., 1996; Semenza, 2000a.) Under conditions of low oxygen, hypoxia inducible factor-1 trans-locates to the nucleus and binds to hypoxia response elements (HREs), unique promoters on genes whose expression is up regulated by hypoxia. Expression of HIF-1 α by macrophages in the rheumatoid synovium has been recently demonstrated (Hollander et al., 2001).

Hypoxic conditions also stimulate glucose transport by four facilitative glucose transporters and prolonged exposure to hypoxia results in enhanced transcription of the Glut-1 gene. Zhang et al. (1999) have extensively reviewed the two mechanisms behind this hypoxic regulation. One mechanism is the stimulation of Glut-1 transcription secondary to the associated inhibition of oxidative phosphorylation during hypoxia and the other is a response to hypoxia-signalling pathways for example HIF-1. Glut-1 expression has already been used to assess tumour hypoxia (Airley et al., 2001.) It has been shown that the Glut-1 gene contains binding sites for HIF-1 (Ebert et al., 1995, Chen et al., 2001). However, a disadvantage for these markers is that their precise affinity for oxygen is not known and so therefore the level of hypoxia cannot be detected. Also hypoxia may not be the only stimulus to induce them. It is known that the pro-inflammatory cytokines, tumour necrosis factor- α (TNF- α) and Interleukin-1 (IL-1) also induce HIF-1 expression (Albina et al., 2001, Thournton et al., 2000).

1.3.2.3 Exogenous Markers of Hypoxia

This technique uses bioreductive compounds which under hypoxic conditions generate reactive metabolites that bind to cellular macromolecules, thus labelling hypoxic cells even if they are subsequently re-oxygenated.

Nitroaromatic compounds have been extensively studied for this type of labelling, the few that have worked successfully *in vivo*, having been based on 2-nitroimidazoles. One of the first, such compounds was isotopically labeled misonidazole. It was found to localize in hypoxic regions in small cell lung cancer and malignant melanomas (Urtasun et al., 1986, Chapman, 1991). However, the use of radioactive isotopes combined with subsequent patient radiotherapy made this method unsuitable for routine clinical use. In 1984 Nitroaryl compounds were considered as potential fluorescent probes for hypoxia. Nitroakridin 3582 is an example of such compounds, which fluoresce only upon reduction. Although Nitroakridin 3582 when incubated with a cell line yielded fluorescence under conditions of reduced oxygen, *in vivo* it was toxic at doses $>0.19 \mu\text{mol}$ (Stratford et al., 1984).

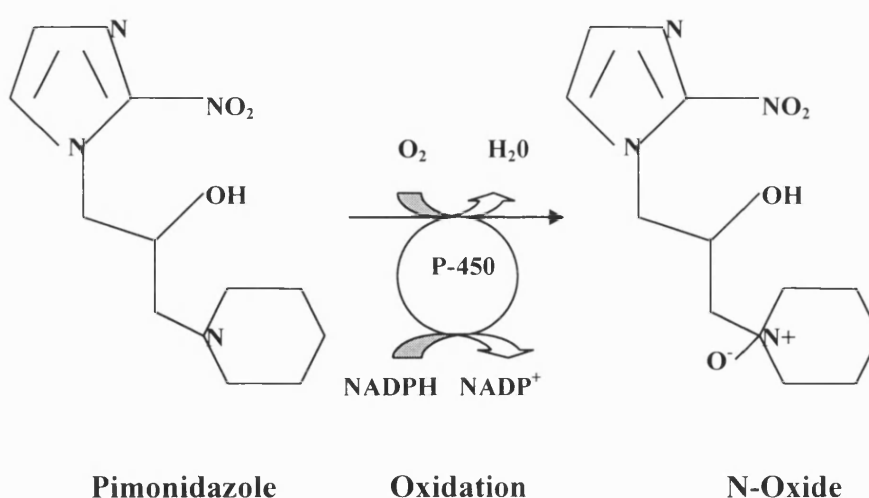
In the 1990's these compounds were superseded by bioreductive compounds detectable using immunological techniques. In 1989 Millar and co workers used polyclonal antibodies raised against a series of radiation chemically produced 2-nitroimidazole-protein adducts in the indirect immunofluorescent detection of hypoxic cells in EMT6/Ed spheroids. They discovered the advantages of this technique: speed, technical simplicity, economy, and independence from the requirement of radiolabeled precursor; thus opening the door for the use in a clinical setting. In 1994 Hodgkiss and co workers synthesized three novel compounds with a 1-substituent containing a biotin moiety. They demonstrated

that bound adducts of these compounds could be identified in hypoxic cells in vitro by the biotin binding proteins, avidin or streptavidin, labeled with fluorescein.

7-(4'-(2-nitroimidazole-1-yl)-butyl)-theophylline (NITP) represents one of the modern generations of these compounds. The bioeductively bound adducts of NITP are identified with a commercial anti-serum to theophylline. In 1994 Hodgekiss and co workers published the pharmacokinetics and discussed various administion methods for this compound. It was found to have limited water solubility and they also suggested that direct site injection was not a suitable method of administration in tumours as the bound metabolites of NITP localized to the injection site (Hodgkiss et al., 1995). This compound has mainly been used to visualize hypoxia in tumors in animal models. One of the disadvantages of this product is that it is not licensed for use in humans. In 1999 Soo used NITP to successfully label hypoxic cells in the rat air pouch pseudosynovial tissue (Days 1-3 post-carrageenan challenge) and inflamed joints.

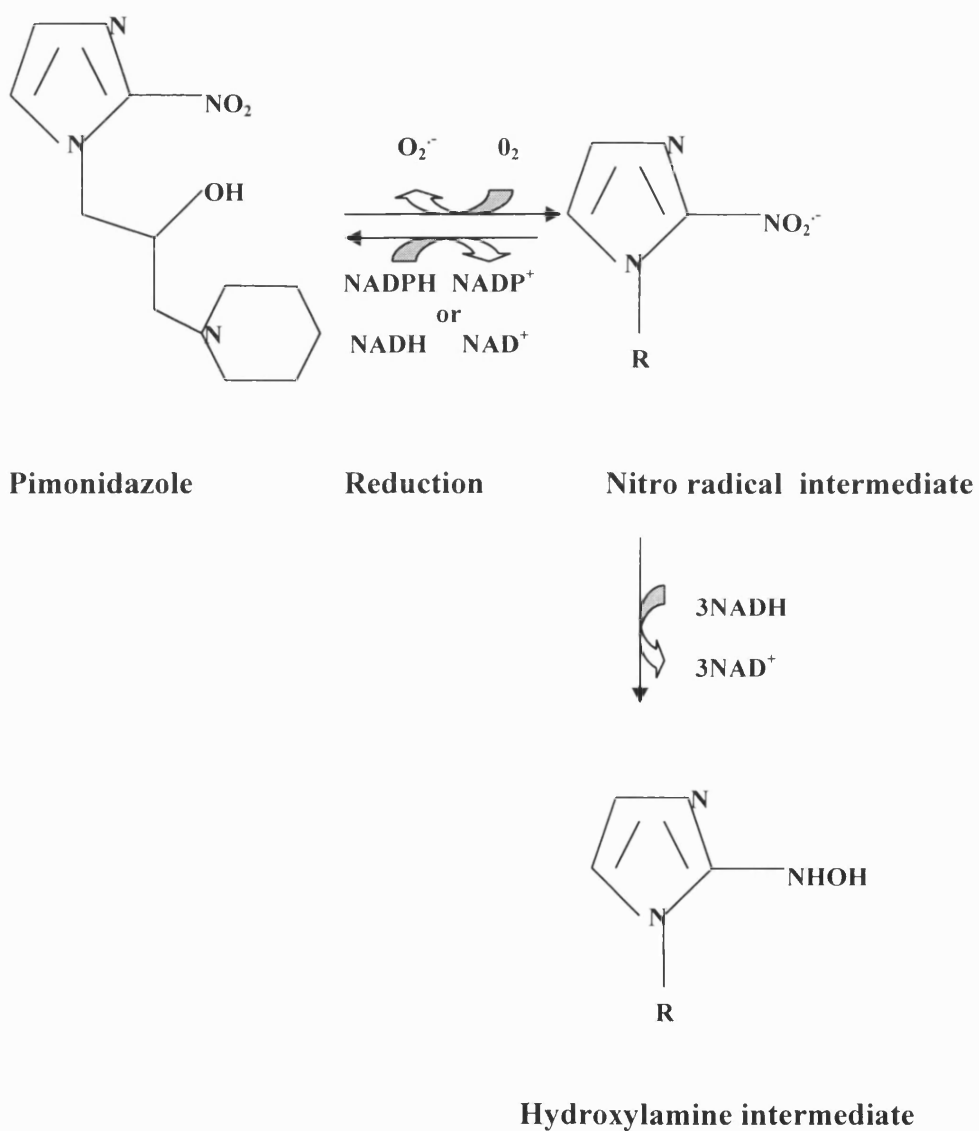
Pimonidazole is a modified 2-nitromidazole, in the presence of oxygen >10mm Hg it is oxidized to pimonidazole N-oxide as in equation 1 below.

Equation 1.



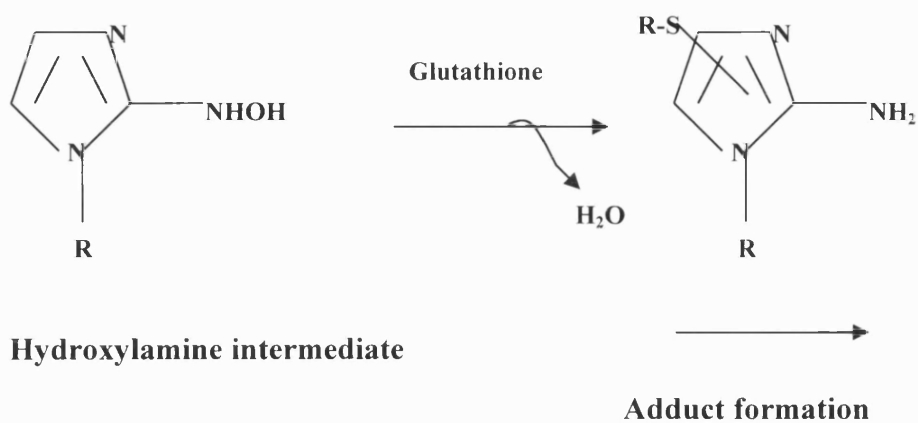
Under conditions of hypoxia pimonidazole is reductively activated nitroreductases transfer electrons to the parent compound as in equation 2 below.

Equation 2.



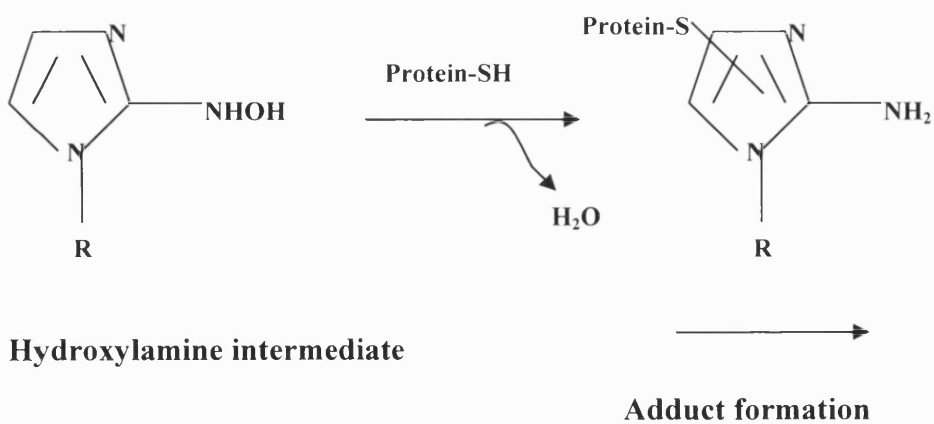
In cells the reduced pimonidazole binds to -SH containing molecules like glutathione as in equation 3 below.

Equation 3.



Alternatively it can bind to proteins and accumulate in the cells, equation 4 below.

Equation 4.



Oxygen inhibits 2-nitromidazole adduct binding by reoxidizing the nitro radical, equation 2 (Arteel et al., 1998).

Cline and co workers (1990) used an immunoperoxidase technique to detect the in vivo binding of a 2-nitroimidazole hypoxia marker in sections of a variety of excised canine tumours. They demonstrated the binding of the marker was restricted to the cytoplasm of the cells. The marker appeared in regions adjacent to necrosis but also in regions free of necrosis. This compound has since gone into commercial production as Hypoxyprobe-1. Hypoxyprobe-1 is an injectable preparation of pimonidazole (a modified 2-nitromidazole) which is taken up by all tissues in the body but binds only to cells with a pO_2 of 10 mm Hg (or less) at 37°C.

Hypoxyprobe-1MAB1 is a monoclonal antibody that binds to protein adducts of Hypoxyprobe produced in hypoxic cells and this allows their detection in tissues *ex-vivo* using a variety of immunohistochemical techniques. 1997 saw its first use in combined immunohistochemical assays detecting hypoxia and proliferation in squamous cell carcinoma of the cervix (Kennedy et al., 1997). Since then Arteel et al (1998) have demonstrated that the reductive metabolism of this hypoxia marker is regulated by oxygen tension independent of pyridine nucleotide redox state. The additional advantages of pimonidazole over the previously mentioned compound are that it is readily soluble in water and is licensed for use in patients. To date there is no published work on the utilization of Hypoxyprobe-1 in studies of inflamed joints either in humans or animals.

1.4. Evidence of Hypoxia in the Rheumatoid Joint from Human and Animal Studies

Many studies have been carried out that demonstrate hypoxia and hypoxic changes in rheumatoid joint but not in non-rheumatoid joints. In 1970, Lund-Oleson examined eighty-five rheumatoid knees and found the mean oxygen tension of the synovial fluid to be 27mmHg. Falchuk et al (1970) observed low partial pressures of oxygen (pO_2), below 30mmHg in RA synovial fluid, combined with decreases in pH and increases in lactic acid concentrations and pCO_2 . Other studies also report on a combination of low pO_2 , increased lactate, low glucose and acidosis (Geborek et al, 1989; James et al, 1990; Wallis et al, 1985a,b). Treuhaft and McCarty, (1971) reported samples with extremely low pO_2 of less than 15.2mmHg in a study of fifty-five samples of RA synovial fluid. More recently Blake et al (1997) used an Eppendorf oxygen probe intra-articularly to directly measure the partial pressure of oxygen in the knees of rheumatoid and non- rheumatoid patients. They showed that only rheumatoid joints had pO_2 measurements in the very low range of 0-15mmHg compared to the normal controls that were in the range of 80-90mmHg.

This work has also been supported by studies in animal models. In 1992 Bruce used electron paramagnetic resonance oximetry to measure the partial pressure of oxygen in vivo in joints of the adjuvant arthritic rat and found a significant decrease in pO_2 between day seven and fourteen post adjuvant arthritis induction when inflammation in the joints was maximal. Claxson and co workers (1999) used high field proton NMR spectroscopy to investigate the metabolic status of rat air pouch exudate after induction of inflammation with carrageenan. They demonstrated substantially elevated levels of lactate, low glucose levels and high levels of the ketone body 3-d-hydroxybutyrate, data consistent with a hypoxic environment and consequent anaerobic metabolism. This reflects reported studies

of RA synovial fluid and sera that also showed increased levels of lactate, ketone bodies and decreased concentrations of glucose using nuclear magnetic resonance analysis (Naughton et al., 1993a,b). In 1999 Soo used 7-(4'-(2-nitroimidazole-1-yl)-butyl)-theophylline (NITP) to successfully label hypoxic cells in the inflamed joints from adjuvant arthritic rats and in rat air pouch pseudosynovial tissue 1-3 days post-carrageenan challenge. Between these time points numbers of NITP positive cells were high in the air pouch reflecting the fact that it is poorly vascularized with consequent metabolic hypoxia in the proliferating pouch wall. This parallels the situation in the RA joint.

1.5 Mechanisms for Hypoxia in the Joint

Two mechanisms may explain why the rheumatoid joint is hypoxic:

- 1.) Inflamed and proliferating tissue has a high metabolic demand but poor neovasculature, which is unable to meet the oxygen demand.
- 2.) High intraarticular pressures resulting from increases in synovial tissue and fluid volume within a rigid capsule causing the collapse of the subsynovial capillary network and a decrease in effective blood flow to the synovium.

The hypoxic environment of the rheumatoid joint shows many metabolic changes these include: lowered glucose levels, raised lactate levels and a decrease in pH in the synovial fluid (Falchuk et al., 1970; James et al., 1992). Additionally, elevated activity of the glycolytic enzymes, glyceraldehyde-3-phosphate and lactate dehydrogenase, in the synovial lining cell population has been detected (Henderson and Glynn, 1981). Detailed analysis of RA synovial fluid and sera by Naughton et al., (1993a,b) using nuclear magnetic resonance (NMR) showed increased levels of lactate and ketone bodies such as 3-D-

hydroxybutyrate and acetoacetate, as well as decreased concentrations of glucose, chylomicron- and very-low-density lipoproteins (VLDL)-associated triacylglycerols in RA synovial fluid and sera. These molecules are all indicative of abnormal metabolic status in the inflamed joint which would require an increase in perfusion and the vascular changes to prevent tissue hypoxia. However, as evidenced in the next section of this chapter the vasculature in the rheumatoid joint is unable to keep pace with the metabolic demands.

1.6 Angiogenesis and Vasculature in the Rheumatoid Joint

Angiogenesis is a complex process that brings about the formation of new blood vessels. It is essential in embryogenesis and wound healing but also occurs in tumours. Many studies consider rheumatoid arthritis (RA) to be an angiogenesis dependent disease (Colville-Nash et al., 1992; Walsh, 1999). This remains a controversial issue, for as will be discussed here, there is evidence for both presence of inhibitors and stimulators of angiogenesis and there are differing views regarding the morphology of vasculature in the rheumatoid joint.

There is a large volume of evidence showing the presence of angiogenic regulators in the rheumatoid joint. Angiogenic stimulators include: Histamine (Frewin et al., 1986), nitric oxide (Farrell et al., 1992, Grabowski et al., 1997), Interleukin-8 (Koch et al., 1992), vascular endothelial growth factor (Koch et al., 1994), and the list continues to grow with new modulators being found in recent years. These include angiopoietins: Ang1 and Ang2 and their receptors, which have key roles in the later stages of angiogenesis (Shahrara et al., 2002). There are also chemokines present in the rheumatoid synovium that have been

shown to stimulate angiogenesis, including synoviocyte-derived CXCL12 (Pablos et al., 2003) and fractalkine (Volin et al., 2001).

Angiogenesis inhibitors are also found including thrombospondin (Koch et al., 1993). These activate cell death in endothelial cells (Armstrong et al., 2003). Other chemokines for example tumour necrosis factor α (TNF α) play the role of inhibitor and stimulator of angiogenesis and this is dependent on the tissue concentration (Farjardo et al., 1992). Perhaps morphometric studies could elucidate whether new blood vessels are being formed in the rheumatoid joint? Two studies looked at cell morphology in the inflamed synovium; they suggested that changes in endothelial morphology to tall endothelial cells or cells resembling high endothelial venules indicated that there was an increase in vascular proliferation in the inflamed synovium (FitzGerald et al., 1991 and Yanni et al., 1993). Other studies which have looked specifically at vessel morphology not cell morphology have produced contrary results. Stevens et al (1991a) observed that the capillary density in RA biopsies was reduced, and with greater spatial distribution compared to normal synovium. Also they observed that the average distance of the capillaries from the joint cavity was increased. Ceponis et al, (1996) found a significant reduction in blood vessels in the superficial synovium compared to non-inflammatory controls and they also observed abnormal Von Willebrand factor (vWF) distribution which they suggested was indicative of either vascular damage and or induced release, perhaps as a result of hypoxia, or reactive oxygen species. Other studies suggest that RA synovial neovascularization may be aberrant, microvessels in the inflamed synovium lacking pericytes and smooth muscle (Walsh, 1999). Blood vessels also lack substance P, calcitonin gene-related peptide and

C- flanking peptide of neuropeptide Y- immunoreactive fibres, neuropeptides involved in vascular regulation (Mapp et al., 1990).

In conclusion the evidence of the presence of angiogenic stimulators clearly shows that the signals for angiogenesis are available. However, the neovascularization is small relative to the amount of tissue and there is all the evidence discussed in 1.4 to show that the rheumatoid joint is hypoxic. This paradox supports the hypothesis that inflamed and proliferating tissue has a high metabolic demand but poor neovasculature, which is unable to meet the oxygen demand.

1.7 High Intra-articular Pressure and Hypoxia

As early as 1967 it was observed by Nakamura et al, that perfusion in the RA joint was poor, with chronic rheumatoid knee effusions having the slowest rates of dye clearance when compared to acutely traumatised joints. Also chronically inflamed joints have been found to possess a higher intra-articular resting pressure than normal joints (Jayson and Dixon, 1970; Lund-Olesen, 1970; Merry et al., 1991; James et al., 1992). Gaffney and co-workers (1995) found the intra-articular pressure in ankle joints of patients with acute traumatic effusions at rest and during exercise was about –3.0 mm Hg, compared to 92.0 mm Hg in patients with chronic effusions. James et al (1990) found that in rheumatoid patients with knee effusions; the increases in intraarticular pressure encountered in normal daily activity could compromise blood flow. Generation of high intraarticular pressures in rheumatoid joints plays a key role in ischaemia-reperfusion injury (also termed hypoxic reperfusion injury), which may also perpetuate synovitis in the rheumatoid joint (James et al., 1992; Edmonds et al., 1993; Halliwell, 1995). The rheumatoid joint cavity has

increased tissue volume due to proliferation of the synovium and increased inflammatory infiltrate. In a small enclosed joint space this tissue becomes further squeezed during exercise. This results in a compression of the synovial capillaries with resultant hypoxia in the joint. When the joint is relaxed, intra-articular pressure decreases sufficiently to allow restoration of blood flow to the inflamed tissue and there is a surge in tissue oxygenation. Repeating this process leads to the inflamed joint being subject to ischaemia-reperfusion cycles. It has been suggested that when reperfusion occurs there is an uncoupling of intracellular redox systems and this leads to the production of reactive oxygen species (ROS) that damage the tissues (Gaffney et al., 1995).

1.8 Ischaemia-Reperfusion Injury Generated Free radicals and Tissue Damage.

A free radical has been simply defined as any species that contains one or more unpaired electrons and is capable of independent existence (Halliwell, 1995). There is a growing body of evidence that suggests that they may contribute to various pathologies. Reactive oxygen species such as superoxide radical, hydrogen peroxide, hydroxyl radical, hypochlorous acid, and reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite, contribute significantly to tissue injury in RA (Bauerova and Bezek, 1999).

As previously discussed in 1.7 it has been suggested that ischaemia-reperfusion injury lead to the production of ROS, which damage tissues in RA. In the 1980's Granger and co workers hypothesised that the enzyme Xanthine oxidoreductase (XOR) played a significant role in the generation of ROS in ischaemia reperfusion injury (Granger et al., 1986). Detailed immunolocalization studies were carried out which found this enzyme in the endothelial cells of a broad range of tissues (Jarasch et al. 1986, Kooij et al., 1992). Of

particular relevance to rheumatoid arthritis is the localization of XOR in the synovial endothelium (Allen et al., 1987, Stevens et al., 1991b). It has also been detected in human mast cells and macrophages (Hellsten-Westing, 1993). In purine catabolism XOR is seen as the terminal enzyme, it catalyzes the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid (Harrison, 2002).

The mammalian XOR enzyme exists in two interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). XDH preferentially reduces nicotinamide adenine dinucleotide (NAD^+), whereas XO reduces oxygen and cannot reduce NAD^+ . Reduction of molecular oxygen by either form of the enzyme yields superoxide and hydrogen peroxide (Harrison, 2002). Granger and co-workers, (1986) theory for the role of XOR in ischaemia-reperfusion injury is that during ischaemia elevated calcium concentrations lead to the proteolytic conversion of XDH to XO and the accumulation of hypoxanthine. When reperfusion occurs XO catalyzes the hydroxylation of hypoxanthine and generates superoxide and hydrogen peroxide. Zhang et al., (1998b) demonstrated that the conversion of XDH to XO was not necessary for post-ischemic ROS generation as both XDH and XO were shown to oxidise NADH. Currently it is accepted that whilst the conversion of XDH to XO may take place it not as important as the overall upregulation of XOR activity (Harrison, 2002). Indeed hypoxia has been shown to increase XOR activity *in vitro* (Poss et al., 1996) and elevated levels of xanthine oxidase have been demonstrated in patients with RA (Miesel et al., 1993).

Normally, ROS produced in the body are removed by antioxidant systems including the enzymes superoxide dismutase and catalase but it has been suggested that during ischaemia-reperfusion cycles, such excessive amounts of ROS are produced that this

defense system is saturated allowing local tissue damage (Halliwell, 1995). Indeed, ROS have been detected in rheumatoid synovial tissue (Singh et al., 1995).

Another means by which XOR could lead to the generation of reactive intermediates is via the generation of nitric oxide (NO). Under conditions of hypoxia XOR can catalyse the reduction of nitrite or nitrate to NO (Miller et al., 1998, Zhang et al., 1998a), and in the rheumatoid joint production of both superoxide and nitric oxide would lead to the interaction of the two and the formation peroxynitrite (ONOO[•]) which is cytotoxic (Radi et al., 1991). The nitration of proteins by RNS such as peroxynitrite can target mitochondrial enzymes, inhibiting cellular respiration and leading to cell death (Radi et al., 1994). Also peroxynitrite may facilitate cartilage damage in the RA joint by inactivating tissue inhibitors of metalloproteinases (Frears et al., 1996, Hadjigogos, 2003).

The XOR catalyzed generation of peroxynitrite has been demonstrated (Godber et al., 2000) and the localization of 3-nitrotyrosine (3-NT), a marker of protein nitration by peroxynitrite (ONOO⁻) and other reactive nitrogen species, has been demonstrated in RA patients synovial tissues (Mapp et al., 2001, Sandhu et al., 2003).

There are several processes by which ROS can damage tissue. Lipid peroxidation products have been found in the synovial fluid (Lunec et al., 1981, Rowley et al., 1984, Dai et al., 1997) and synovial membrane (Winyard et al., 1993) of RA patients. Lipid peroxidation of cellular membranes and lipoproteins occurs when a free radical reacts with a polyunsaturated fatty acid, setting off a chain reaction of oxidizations and forming by-products that are cytotoxic or capable of inhibiting cell function (Hessler et al., 1983, Morel et al., 1983).

As well as tissue damage it has been suggested that ROS may perpetuate synovitis by stimulating proliferation of RA fibroblasts, ROS such as hydrogen peroxide, have been found to modulate fibroblast proliferation in culture (Murrell et al., 1990, Burdon, 1995; Burdon 1996).

Amino acids can also be damaged by ROS and RNS and this has led to the theory proposed by Lunec in 1985 that autoantigens are formed by free radical damage to IgG. Indeed, free radical altered IgG in RA patients was found to be higher than normal controls (Blake et al., 1985). More recently Uesugi and co-workers (2000) demonstrated the modification of IgG after exposure to hypochlorous acid (HOCl) and peroxynitrite (ONOO). They also showed rheumatoid synovial fluids contained significantly greater concentrations of nitrosated and chlorinated IgG compared with osteoarthritic specimens.

Apart from damaging tissue by the formation of free radicals via the ischaemia-reperfusion cycle, hypoxia can have other notable effects at a cellular level, which will be discussed in the next section.

1.9 The Effects of Hypoxia in Rheumatoid Arthritis

1.9.1 Hypoxia and cytokines

The importance of the role cytokines play in the pathology of rheumatoid arthritis cannot be over emphasised. Leukocytes and other cells in the inflamed joint produce inflammatory mediators which include cytokines and chemokines (Harris 1990). Chemokines are chemotactic cytokines which exert their effect upon neutrophils, lymphocytes and monocytes leading to their recruitment, activation and persistence in the

synovial tissue. At the time of writing chemokines are divided into four families based upon the location of two conserved cysteine residues: C-X-C, C-C, C, and CX3-C. The cytokine is considered to be a ligand and so is therefore given a ligand (L) designation. Cytokine receptors (R) exhibit non-specific affinity for their ligands (Szekanecz et al., 2003).

A number of cytokines can be evidenced that support the hypothesis that hypoxia stimulated cytokines may have an important role in the pathology of RA. These include a member of the C-X-C family CXCL8 or interleukin-8 (IL-8), elevated levels of which have been found in the joints of RA patients (Endo et al., 1991). IL-8 regulates leukocyte adhesion molecules in RA (DeGendt, 1996) and studies on endothelial cells exposed to hypoxia have shown enhanced expression of this cytokine (Karakurum et al., 1994). Hypoxia followed by reoxygenation has been shown to stimulate IL-1 expression in monocytes (Koga et al., 1992) and increased expression of IL-1 has been demonstrated in human endothelial cells exposed to hypoxia (Shreenivas et al., 1992). IL-1 together with TNF α can stimulate the production of IL-8 by synovial fibroblasts (Hosaka et al., 1994). Both TNF- α and IL-1 can stimulate the release of matrix metalloproteinases (MMPs) by synovial fibroblasts and chondrocytes (Abramson et al., 2002).

Another member of the C-X-C cytokine family, CXCL12 or stromal cell-derived factor 1 (SDF-1) promotes migration and activation of B lymphocytes and hypoxia has been shown to induce SDF-1 in parallel with vascular endothelial growth factor (VEGF) (Pablos et al., 2003).

Hypoxia/reperfusion has been implicated in the production of a number of other cytokines; TNF α (VanOtteren et al., 1995) stimulating the production of many other

pro-inflammatory factors involved in rheumatoid arthritis and will be discussed in more detail later on. Basic fibroblastic growth factor (bFGF), is a potent mitogen and angiogenic factor which simulates growth in many cells including various tumour cell lines (Goddard et al., 1992) and could be responsible for inducing the transformed appearance of the rheumatoid cells and transforming growth factor β (TGF- β), important because it modulates fibroblast recruitment and proliferation (Berse, 1999).

1.9.2 Hypoxia and Matrix Metalloproteinases (MMPs)

Articular cartilage possesses unique structural properties that make it particularly suited to a role in protecting the bone heads from damage. In rheumatoid arthritis proteolytic degradation of the extracellular matrix (ECM) of cartilage by matrix metalloproteinases (MMPs) has been identified as a key step in joint destruction (Nagase and Woessner, 1999, Okada, 2000). Matrix metalloproteinases are a gene family of zinc metalloproteinases that can degrade ECM components. Collagenases (MMP 1, 8 and 13) degrade collagen, stromelysins (MMP 3, 10 and 11) degrade proteoglycans and activate latent collagenases. Gelatinases (MMP 2 and 9) further degrade collagen that has already been clipped by collagenase. MMPs are composed of three well-conserved domains: a propeptide domain, a catalytic domain and a hemopexin-like domain (Massova et al. 1998). They are secreted from the connective tissue cells such as fibroblasts, myoblasts, chondrocytes, osteoblasts, synovial cells, endothelial cells and inflammatory cells, including macrophages, monocytes, neutrophils and mast cells (Turto et al. 1977, Emonard and Grimoud 1990, Tozzi et al. 1998, Johnson et al. 1998). Matrix metalloproteinases such as collagenase 1 (MMP-1), stromelysin-1 (MMP-3) and gelatinase B (MMP-9) have been

shown to be elevated in patients with RA (Vincenti et al., 1994, Klimiuk et al., 2002). MMP enzymatic activity is regulated at four levels: gene transcription, secretion, pro-enzyme activation and enzyme inhibition by tissue inhibitors of metalloproteinases (TIMPs). It has been proposed that it is an inequality in the TIMP to MMP ration that results in diseases like arthritis, associated with uncontrolled proteolysis of connective tissue (Gomez et al. 1997). Indeed inactivation of TIMP-1 by peroxynitrite has been demonstrated (Frears et al., 1996).

MMPs are synthesized in an inactive pro-enzyme form then converted to the active enzyme. In cardiovascular research it has been proposed by Novotna et al (2002) that activation of collagenolysis in the early phases of hypoxic exposure is related to the production of reactive oxygen species (ROS). This is of particular relevance to rheumatoid arthritis; where ROS and RNS have been demonstrated (see section 1.8.). Superoxide, hydrogen peroxide, nitric oxide and peroxynitrite all react with the catalytic site of the metalloproteinases (Rajagopalan et al. 1996), it has been shown experimentally that peroxynitrite activates MMP-8 (Okamoto et al. 1997), proMMP-1 and proMMP-9 (Okamoto et al. 1997) and MMP-2 and MMP-9 (Rajagopalan et al. 1996). A secondary route by which hypoxia could stimulate the release of MMPs is through the cytokine IL-1. As mentioned in section 1.9.1 hypoxia followed by reoxygenation has been shown to stimulate IL-1 expression in monocytes (Koga et al., 1992) and increased expression of IL-1 has been demonstrated in human endothelial cells exposed to hypoxia (Shreeniwas et al., 1992). IL-1 activates the release of MMPs by chondrocytes and by synovial fibroblasts at the cartilage-pannus interface (Abramson et al., 2002).

1.9.3 Hypoxia and Other Enzymes

As previously mentioned in section 1.8 a key enzyme found in RA synovium and whose activity is upregulated by hypoxia is xanthine oxidoreductase (XOR). XOR can generate tissue damaging reactive species, superoxide and hydrogen peroxide and under conditions of hypoxia has the capacity to form peroxynitrite a reactive nitrogen species also capable of detrimental effects within cells.

Another enzyme with the capacity to produce reactive intermediates is inducible nitric oxide synthase (iNOS). iNOS is one of three isoforms of nitric oxide synthase the others being neuronal form (nNOS) and endothelial form (eNOS). iNOS synthesises nitric oxide (NO) from the catalytic conversion of L-arginine to L-citrulline via a chemical reaction between the guanidino-nitrogen of L-arginine, dioxygen and NADPH. (Gross et al., 1995). The activity iNOS can be induced by lipopolysaccharide (Xie et al., 1994) hypoxia (Melillo et al., 1995) and pro-inflammatory cytokines such as IL-1, IFN- γ and TNF α (Wu, 1995). Also the mRNA initiation site of the iNOS gene has a promoter and two binding regions for various transcription factors including nuclear factor kappaB (NF- κ B) (Xie et al., 1994). iNOS is highly expressed in the rheumatoid synovium, particularly in synovial fibroblasts (Sakurai et al., 1995, Gabowski et al., 1997). NO production is seen in macrophages, neutrophils and mast cells (Stuehr et al., 1987, McCall et al., 1991, Salvemini et al., 1990). Also NO generation has been demonstrated in RA synovial cultures (McInnes et al., 1996) and this work is supported by animal models - adjuvant arthritis and collagen-induced arthritis, which show an increase in iNOS messenger RNA and increased nitrate urinary excretion (Cannon et al., 1996).

NO may also have an anti-inflammatory function by preventing the adhesion and release of oxidants by activated neutrophils in the microvasculature (Abramson et al., 2001), but for the most part is considered pro-inflammatory. It can, however, be seen as having two roles in rheumatoid arthritis. There is the previously described role in the generation of tissue damaging reactive nitrogen species, by combining NO with superoxide to form peroxynitrite, (section 1.8). Peroxynitrite can target mitochondrial enzymes, inhibiting cellular respiration and leading to cell death (Radi et al., 1994) and may facilitate cartilage damage in the RA joint by inactivating tissue inhibitors of metalloproteinases (Frears et al., 1996, Hadjigogos, 2003).

A subtler role for NO in cell signalling has also been recognised. NO was originally identified as endothelium derived relaxing factor (Palmer et al., 1987) and this is indicative of its role in the regulation of vascular tone. In rheumatoid arthritis this would enable an increase in blood flow at the site of inflammation. It has been shown that apoptosis can be induced by NO in cultured human fibroblasts and is associated with nuclear p53 protein expression (Borderie et al., 1999, Van't Hof et al., 2000). Finally NO can also induce the expression of two other enzymes associated with RA, cyclooxygenase-2 (COX)-2 and haemoxygenase-1 (HO)-1 (Salvemini et al., 1993 Foresti et al., 1997).

The enzyme cyclooxygenase (COX) mediates prostaglandin (PG) production from arachidonic acid (AA) (Smith et al., 1991). Prostaglandins have been found in synovial tissue and fluid by many researchers (Egg, 1984, Henderson et al., 1987). The presence COX in rheumatoid synovium was observed in 1992 by Sano et al; and prostaglandin production has been shown to be maintained in hypoxic rheumatoid fibroblasts (McGough et al., 1997). Two isoforms of COX exist; COX-1 a constitutively expressed enzyme and

COX-2 the inducible form involved in inflammatory responses. Synovial tissues from patients with RA are shown to contain COX-2 and to a less extent COX-1 (Trang et al., 1977). COX-2 is stimulated by the same stimulatory factors as iNOS namely, hypoxia (Schmedtje, 1997 Cernanec et al, 2002), IL-1, TNF- α , IFN- γ . Like iNOS, COX-2 has an NF- κ B binding site, (Wu, 1995), and this will be discussed further in section 1.9.4. Peroxynitrite has been linked to the signalling pathways that induce COX-2 (Migita et al., 2002). Prostaglandins play an important role in rheumatoid arthritis as they can increase vascular permeability, oedema and pain in synovial tissues and as early as the 1970's there was evidence that PGs are mediators involved in the development of erosions of articular cartilage (Robinson et al., 1975, Henderson et al., 1987).

Haem oxygenase (HO) like cyclooxygenase exists in two isoforms, inducible HO-1 and constitutively expressed HO-2. HO catalyzes the cleavage of haem to biliverdin and carbon monoxide, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) (Maines, 1988). HO-1 is recognized as the 32kDa heat shock protein (HSP) (Keyse et al., 1989). HSPs are highly conserved proteins classed by their molecular weight and are induced by stressful conditions like sudden changes in temperature or oxidative stress, they are a cells survival technique, a mechanism by which they might adapt to a changing environment. HO-1 can be induced in fibroblasts and other cell types by hypoxia, free radicals (Panchenko et al., 2000) and cytokines: IL-1 and TNF α (Cantoni et al., 1991). It carries binding sites for AP-1 and NF κ B transcription factors (Lavrovsky et al., 1994). Research into HO-1 in has been identified in arthritic synovia (Winrow et al., 1995). In normal bone, induction of HO-1 mRNA has been demonstrated following physiological levels of mechanical loading in vivo (Rawlinson et al., 1998). In a rat model of carrageenin

induced acute inflammation HO induction brought about the resolution of the inflammation (Willis, 1995) HO-1 has also been shown to prevent cell death by virtue of its capacity to cleave heme to biliverdin which has antioxidant properties (Stocker et al., 1987). Finally, there is a regulatory loop between HO-1, COX and iNOS: Carbon monoxide produced by HO-1 can inhibit iNOS and COX (White et al., 1992). While NO conversely, can induce HO-1 and COX-2 (Salvemini et al., 1993 Foresti et al., 1997).

1.9.4 Hypoxia and Gene Control

The responses of a cell to changes in the local environment ultimately lie at gene level. Each gene has a promoter with binding regions for transcription factors, the end result being the production of a specific gene product.

Certain transcription factors are activated by oxidative stress caused by hypoxic reperfusion events. One transcription factor specifically activated by hypoxia has previously been mentioned in 1.3.2.2. The HIF-1 transcription factor is a dimer composed of 2 subunits belonging to the helix-loop-helix-per-arnt-sim (bHLH-PAS) protein family. The subunits are termed HIF-1 α and HIF-1 β (Semenza 1999). The HIF-1 α subunit is oxygen regulated and determines HIF-1 activity (Jiang et al., 1996; Semenza, 2000.) Under conditions of low oxygen, HIF-1 α protein becomes stabilised and is able to bind to HIF-1 β to form a transcriptionally active complex (Salceda et al., 1997). The active HIF-1 complex translocates to the nucleus and binds to hypoxia response elements (HREs), unique promoters on genes whose expression is up regulated by hypoxia. There are a number of genes that are regulated by HIF-1, those of particular relevance to rheumatoid arthritis being: iNOS, VEGF and PDGF (Ratcliffe et al, 1998). Also, with the recent development

of commercial antibodies to HIF-1 α , expression of HIF-1 α by macrophages in the rheumatoid synovium has been demonstrated (Hollander et al., 2001).

Nuclear factor kappa B (NF κ B) has been long established as a transcription factor that responds to oxidative stress (Schreck et al., 1992, Haddad, 2002) and hypoxia (Koong et al., 1994). It has been localised in the vascular endothelium and type 'A' synovial cells from RA patients (Marok et al., 1996), a finding which has been supported by studies in adjuvant arthritis that localized NF κ B to the intimal lining of the synovium (Tsao et al., 1997). In rheumatoid arthritis NF κ B mediates expression of many inflammatory factors including TNF α (Foxwell et al., 1998), IL-2 and cell adhesion molecules ICAM-1 (Collins et al 1995), VCAM-1 (Baeuerle et al., 1997, Manning et al., 1995) and E-selectin (Chen et al., 1995). NF κ B is a heterodimer that exists in the cytoplasm in an inactive form, with critical binding sites masked by an inhibitory protein (I κ B). Upon activation a signal cascade results in the degradation of the inhibitory proteins enabling NF κ B to translocate to the nucleus binding to target genes to be expressed (Allen and Tresini, 2000). Recently, ICAM-1 upregulation as a result of NF κ B activation by hypoxia/reoxygenation in synovial fibroblasts has been demonstrated, implicating hypoxia reoxygenation in the sequestering of lymphocytes in the inflamed joint (Han et al., 2003). iNOS, COX-2, and HO-1 possess binding sites for NF κ B (Xie et al., 1994, Wu, 1995, Lavrovsky et al., 1994), confirming the key role NF κ B plays in perpetuating inflammation in the rheumatoid joint.

Activator protein-1 (AP-1) is another transcription factor expressed in the rheumatoid synovium (Handel et al., 1995), stimulated by a variety of inflammatory factors including cytokines TNF α and IL-1 (Firestein et al., 1990), growth factors and oxidative stress (Willoughby and Tomlinson, 1999). AP-1 consists of heterodimers or homodimers of

Jun- and *-Fos*. AP-1 mediates T cell activation and production of cytokines and matrix metalloproteinases (MMPs) (Angel et al., 1991).

Hypoxic reperfusion injury and the resulting formation of free radicals has previously been discussed with regard to rheumatoid arthritis in section 1.8. Transcription factor, p53 tumour suppressor gene (p53) can be induced by reactive oxygen metabolites (Hansson et al., 1996). Over expression of p53 has been observed in RA synovium (Firestein et al., 1996) and mutations of the gene have been found associated with synovial neoplasia (Han et al., 1999). In 1998 Aupperle et al., observed that inactivation of p53 protein decreased apoptosis in fibroblast-like synoviocytes thus attenuating their proliferation.

1.9.5 Hypoxia and Bone Remodelling

'Bone remodelling' is a continuous process of renewal and repair carried out by two cell types: osteoclasts, which re-absorb bone, and osteoblasts, which form new bone. Old or damaged bone is removed by osteoclasts by the secretion of acid and proteolytic enzymes on to the bone surface. Subsequently the osteoclasts migrate away from the area of bone undergoing resorption and undergo apoptosis. They are replaced by osteoblasts, which lay down new bone matrix.

In rheumatoid arthritis focal bone erosions occur at the joint margins and in subchondral bone. The site of bone erosion contains cells with phenotypic characteristics of osteoclasts, (Takayanagi et al., 1997). These erosions have been correlated with disease severity (Goldring, 2002) and analysis of markers for bone metabolism in early stages of

RA show an increase in osteoclast-mediated resorption that is not matched by new bone formation (Goldring et al., 2000).

As previously discussed in section 1.9.3 iNOS is an enzyme that can be induced by hypoxia (Melillo et al., 1995) and synthesises nitric oxide. There is evidence to suggest that NO effects osteoclastic bone resorption. *In vitro* studies have shown that low concentrations of NO potentiate IL-1 induced bone resorption and high concentrations inhibit bone formation (Ralston et al., 1995). The interactions of NO with free radicals may attenuate bone erosion as superoxide and hydrogen peroxide can stimulate osteoclastic bone resorption (Hadjigogos, 2003) NO stimulates the production of matrix metalloproteinases in chondrocytes, which may lead to further destruction of the joint (Murrell et al., 1995).

Receptor activator of nuclear factor- κ B (RANKL) is a membrane-bound TNF-related factor that is expressed by osteoblast/stromal cells. The presence of RANKL is vital for osteoclast differentiation (Suda et al., 1999). Recently Takayanagi et al. (2000) have shown that RANKL mRNA is highly expressed in synovial tissues from patients with RA, but not in OA synovial tissues. This expression has been demonstrated in synovial fibroblasts, as well as in activated T cells derived from RA synovial tissues, suggesting that these cells may contribute to osteoclast formation at the specific sites of bone destruction in rheumatoid arthritis (Horwood et al., 1999).

1.9.6 Concluding remarks.

The expression of many of the hypoxic activated inflammatory factors and transcription factors discussed in this section have been evidenced in the histopathology of

rheumatoid arthritis discussed in section 1.2.5 lending strong evidence to support a hypothesis that inflammation in rheumatoid arthritis may in part be driven by hypoxia resulting from poorly vascularized but highly proliferative tissue. The next section will look at the current pharmacological therapies prescribed by conventional medicine today and take a brief look at the new frontier therapies.

1.10 Treatment of Rheumatoid Arthritis

The wide range of therapies for rheumatoid arthritis (RA) include: surgery, physiotherapy, diet supplementation and combinations of drugs. However, for the purpose of this thesis the content in this section will concentrate on single drug therapies. Some of these have been around for years and can be divided into four groups based upon their mode of actions:

1. Analgesics
2. Non-steroidal Anti-inflammatory Drugs (NSAIDs)
3. Corticosteroids
4. Disease Modifying Anti-rheumatic Drugs (DMARDs)

Recently, however, expanding knowledge of the molecular interactions within RA has lead the development of new therapies and these will be briefly browsed under the section of 'Therapies from research and development', they include:

5. Therapies targeting hypoxia
6. Anti cytokine therapy
7. Angiogenesis inhibitors
8. Gene therapy

1.10.1 Analgesics

Analgesics are used commonly in the treatment of RA. They include peripheral (non-narcotic) analgesics like acetaminophen (paracetamol) and the centrally acting (narcotic) analgesics such as codeine. The mode of action of paracetamol still remain elusive, it is known that its antipyretic actions arise from the inhibition of prostaglandin synthesis in the hypothalamus (Firestein et al., 2000). The advantage of using paracetamol is it lacks the gastric toxicity associated with many non-steroidal anti-inflammatory drugs. However, in overdose levels paracetamol can lead to hepatotoxicity. The use of codeine and other opioids is primarily for acute pain and is not suited to chronic pain management. This is because of the risk to the elderly posed by the associated side effects of, constipation, drowsiness and impaired cognitive function (Firestein et al., 2000).

1.10.2 Non-steroidal Anti-inflammatory Drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of RA based on their ability to reduce inflammation. All NSAIDs are peripheral analgesics and provide some pain relief on account of their anti-inflammatory effects. Acetylsalicylic acid (aspirin) originally derived from willow (salix) bark, is one of the best known NSAIDs.

NSAIDs have a common chemical structure which means that their mode of action is similar. They are essentially weak acids and function as inhibitors of cyclooxygenases (Vane, 1971) which catalyse production of prostaglandins (a process discussed in section 1.9.2). Prostaglandins cause pain and oedema by enhancing the effects of other mediators such as bradykinin and histamine (Ferreira, 1981). Therefore by inhibiting

cyclooxygenases, pain can be blocked locally. They also reduce the swelling and increased temperature of inflamed joints (Abramson, 1989). However, these drugs are also associated with adverse side effects, involving the neurological, renal, cardiovascular and gastro intestinal systems. Migraines and impaired cognitive function have been associated with indomethacin (O'Brien et al., 1985) and kidney tissue damage with many NSAIDs (Clive et al., 1984). Blood pressure increases which can be additive to pre-existing cardiac problems (DeLeeuw, 1996) and erosions induced in the upper gastrointestinal tract by an increased gastric acid secretion as a result of local prostaglandin inhibition (Ferreira et al., 1979).

Newer NSAIDs such as etoricoxib and valdecoxib have been developed. These aim to selectively inhibit COX-2, which is predominantly induced in pathological states and therefore these drugs should have fewer side effects that result from the additional inhibition of COX-1. Recently, etoricoxib has been approved for treatment of RA patients. Clinical trials show this drug has the equivalent clinical efficacy of traditional NSAIDs without effecting COX1 activity (Patrignani et al., 2003).

It should be noted that NSAIDs reduce inflammation and provide pain relief but disease progression is not prevented. Indeed, NSAID therapy has been associated with cartilage damage (Rashad et al., 1989).

1.10.3 Corticosteroids

Humans synthesize the glucocorticoid, cortisol (hydrocortisone) as a response to stress. The effects of glucocorticoid are wide ranging and explain why synthetic administered glucocorticoids are useful in the treatment of rheumatoid arthritis. Glucocorticoids bind to specific receptors that are located in the cytoplasm. Once bound,

the ligand-receptor complex can enter the nucleus where it binds to regulatory DNA sequences on genes that respond to glucocorticoids. Glucocorticoids suppress inflammation as a result of their ability to interfere with transcription factors NF κ B, AP-1 (Cato et al., 1996) (previously mentioned in section 1.9.3.) and the expression of multiple inflammatory genes including: IL-1, IL-2, IL6, TNF- α (Grabstein et al., 1986, Buttgeriet et al., 1995). Glucocorticoids can also inhibit the trafficking of neutrophils, macrophages and monocytes (Firestein et al., 2000). Synthetic glucocorticoids like prednisolone have therefore been developed and used in the treatment of RA. Although they are good anti-inflammatories they have not been found to inhibit the progression of RA, and also have side effects. Little is known about the molecular mechanism involved in the side effects, which include suppression of endogenous cortisol production in patients given oral corticosteroids or intraarticular steroid therapy (Jasani et al., 1967, Hupertz and Pfuller, 1997). This could leave these patients at risk of acute adrenal insufficiency and abnormal responses to stress. Long term usage has also been associated with induction of osteoporosis (Nielsen et al., 1988). Also, if there is systemic inhibition of key inflammatory cells, then a patient undergoing glucocorticoid therapy is at risk of opportunistic infection from agents like staphylococci, fungi and viruses.

1.10.4 Disease Modifying Anti-rheumatic Drugs (DMARDs)

Disease Modifying Anti-rheumatic Drugs (DMARDs) were once seen as a 'last line of defense' for when rheumatoid arthritis did not respond to conventional drug therapy. However, as more of these agents are discovered and as the knowledge of RA pathology

increases, they are increasingly used in therapy combinations and earlier on in the disease course. DMARDs include: chloroquine, cyclosporine, gold salts, and various immunosuppressive agents, they are slow-acting, their effects being seen after prolonged administration for 3-6 months.

The observation that antimalarials such as chloroquine and hydroxychloroquine induce remission of RA was an incidental finding in RA patients that were using antimalarials for other reasons. (Pybus, 1982;1984; 1985). Their mode of action is thought to be due to the inhibition of antigen processing by macrophages and monocytes (Fox, 1995). Another drug whose anti-rheumatic properties were incidental to a prime use in organ transplantation is cyclosporin. Its mode of action is through the inhibition of IL-2 production and T-cell activation (Bentin, 1995).

Gold compounds such as aurothioglucose, sodium aurothiomalate and auranofin act by suppressing immune responsiveness at a humoral and cellular level and thereby stopping the progress of active arthritis. Other immunosuppressive agents include azathioprine, methotrexate, leflunomide, and cyclophosphamide. Methotrexate, is particularly effective in treating patients with longstanding severe RA. Studies showed that patients were able to tolerate low doses of the drug over prolonged intervals (Willkens and Watson, 1982). More recently it has been used in combination with anti-TNF therapy (Maini et al., 1998).

Thalidomide is an anti-angiogenic and immunomodulatory agent, it has been considered for the treatment of RA, although it's well known side effects of teratogenesis require a great deal of caution in its administration. Its mode of action is not fully understood, but it has been shown to suppress tumour necrosis factor alpha (TNF- α) production and to modify the expression of TNF- α -induced adhesion molecules on

endothelial cells and on human leukocytes (Ossandon et al., 2002). Several open clinical trials have produced conflicting results. Keesal et al (1999) used thalidomide to treat refractory rheumatoid arthritis, they observed no significant improvement in any outcome measure. Other studies of thalidomide in combination with pentoxifylline have shown clinical improvement (Huizinga et al 1996). It seems probable that this drug is not likely to be widely administered, as positive outcome is out-weighed both by known historical toxicology and more recent reported adverse side effects, amenorrhea, peripheral sensory neuropathy drowsiness, constipation, and oedema of the lower limbs (Gutierrez-Rodriguez et al., 1984, 1989).

All of the agents described in this section have potentially serious or even lethal side-effects. They tend to be used in patients with severe active disease with erosions occurring early in the course of the disease, where there is little or no response to intensive NSAID therapy (Klemp, 1983).

1.10.5 Therapies from research and development: Targeting hypoxia

Some of the first therapies to target hypoxic tissue in oncological disease were bio-reductive drugs. These pro-drugs are metabolised to either generate or release an active species in the presence of reducing environment and are therefore preferentially cytotoxic towards hypoxic cells. There are three main classes of bio-reductive drugs: quinones, nitro compounds and benzotriazine di-N-oxides. The toxicity of quinones results from their one electron reduction to a semi-quinone free radical that covalently interacts with and damages DNA. Nitro prodrugs are reduced by a series of single electron transfers generating an

reactive species. Benzotriazine di-N-oxides are also reduced to a reactive nitrogen species. The N-oxide free radical is thought to act as an oxidising radical that abstracts hydrogen from the bases within DNA (Kaye et al., 2001).

Endogenous reducing enzymes implicated in bioreductive drug activation include NADP(H) oxidoreductase, NADPH cytochrome c (P450) reductase, cytochrome b5 reductase, carbonyl reductase and xanthine oxidase/xanthine dehydrogenase (Jaffar et al., 2001). These enzymes act to varying degrees and at different stages in a given prodrug activation.

Bioreductive drugs are often used in combination with radiotherapy in the treatment of cancer. One of the first to be developed was the quinone-alkylating agent, mitomycin C which has been widely used in combination chemotherapy regimens for breast cancer (Kaye et al., 2001). Tirapazamine, a bioreductive drug of the N-oxide class, has been found to be highly efficient in killing hypoxic cells and is extremely active when used in combination with fractionated radiation schedules (Adams and Stratford, 1994). Prodrugs are also used in treatment of bacterial infections because they can demonstrate selective toxicity for microorganisms possessing specific activating enzymes. Nitazoxanide (NTZ) is a redox-active nitrothiazolyl-salicylamide prodrug that kills *Helicobacter pylori* (Sisson et al., 2002). It has been suggested that the specific bioreductive therapy could be used in the treatment of rheumatoid arthritis by combining bioreductive therapy with hypoxia regulated gene therapy, where the therapeutic gene encodes an enzyme involved in the reductive metabolism of the drug of interest (Jaffar et al., 2001).

1.10.6 Therapies from research and development: Anti Cytokine therapy

Throughout this introduction and in section 1.9.1 in particular the importance of cytokines in the pathology of rheumatoid arthritis has been discussed. It has been stated that there is a predominance of pro-inflammatory cytokines in the inflamed joint and this has led to the development of a new concept, cytokines as a pharmacological target for RA therapies. Out of this concept two therapeutic targets dominate the current research: TNF- α and IL-1. In RA they both promote inflammation by stimulating the production of other pro-inflammatory cytokines (Feldmann et al., 1996) and the release of degradative enzymes at the cartilage-pannus interface (discussed in section 1.9.1). IL-1 also stimulates the production of nitric oxide and prostaglandinE2 (PGE2) from articular chondrocytes (Amin, 2000); mediators indicated in section 1.9.3 to contribute to attenuation of inflammation in RA.

Anakinra is a recombinant human IL-1 receptor antagonist (IL-1Ra) that binds to type 1 IL-1 receptors but does not stimulate any intracellular responses. Controlled studies in patients with RA treated with anakinra have led to the conclusion that IL-1Ra is clinically effective and that it slows progression of bone damage as measured radiographically (Bresnihan et al., 1998).

To date, two anti-TNF agents, infliximab and etanercept, have been approved for use in RA treatment, each having a different mode of action. Infliximab is a chimeric anti-TNF- α monoclonal antibody containing a murine TNF- α binding region and human IgG1 backbone. Etanercept is a recombinant fusion protein of the soluble type II TNF receptor on a human IgG1 backbone. Both agents bind to TNF- α released in the inflamed joint and therefore prevent it from interacting with TNF- α receptors on target cells. Clinical

trials of these agents demonstrate efficacy and repeated administration resulted in a sustained reduction in symptoms and signs of RA in the majority of patients (Elliot et al., 1994, Weinblatt et al., 1999) Combination with methotrexate also appears to be effective (Maini et al., 1998). However, as with all drugs anti TNF therapy is not without concerns about potential adverse side effects. This is born out by a study that has linked anti TNF therapy to neurologic events suggestive of demyelination (Mohan et al., 2001) and as with all agents that suppress the immune system there is an increased risk of opportunistic infection. Therefore patient screening should be carried out to avoid the reactivation of latent *Mycobacterium tuberculosis* infection (Criscione et al., 2002). The other disadvantages of this therapy are it is very expensive and 20-40% of patients are “non-responders”. This has been linked to genetic polymorphisms that influence the balance of inflammatory cytokines (Padyukov et al., 2003).

The frontier of anti cytokine therapies looks set to expand exponentially as other studies bring new targets to light. For example: the cytokines driving T-cells IL-12 and IL-15 and targeting cell recruitment through adhesion molecules like ICAM-1 (Haraoui et al., 2000). Vascular endothelial growth factor (VEGF) has also been targeted with anti-cytokine therapy, but this will be discussed in the next section.

1.10.7 Therapies from research and development: Angiogenesis Inhibitors.

As discussed in section 1.7 many studies consider rheumatoid arthritis to be angiogenesis dependent. Although this remains a controversial issue, it has lead to the concept of a new therapeutic target with the suggestion that modulation of angiogenesis in

rheumatoid arthritis would restrict growth of the invasive pannus tissue and thus reduce subsequent cartilage and bone damage (Walsh, 1999).

Some disease modifying anti-rheumatic drugs (DMARDs) including bucillamine (BUC), gold sodium thiomalate (GST), methotrexate (MTX) and salazosulfapyridine (SASP) have been shown to inhibit vascular endothelial growth factor (VEGF) in synovial fibroblasts (Nagashima et al., 1999). As a cytokine VEGF is considered a potent inducer of angiogenesis (Brenchley, 2000). COX-2 inhibitors have also been shown to inhibit angiogenesis through their suppression of VEGF (Jones et al., 1999).

Some of the first reports of an agent that specifically inhibited neovascularization in animal models of arthritis were for AGM-1470 (TNP-470) (Peacock et al., 1992). This agent is toxic to endothelial cells. Inhibition of $\alpha v/\beta 3$ integrin has also demonstrated efficacy in a rabbit model of arthritis (Storgard et al 1999) and endostatin, an anti angiogenic drug used in the treatment of tumours, showed a dose dependent decrease in inflammatory cells and a reduction of von Willebrand factor VIII and type IV collagen positive vessels in RA tissue grafted SCID mice (Matsuno et al., 2002).

Additionally, VEGF has been seen as a target for modern anti-angiogenic therapy in cancer research. Several synthetic VEGF receptor kinase inhibitors have been developed, of which PTK787/ZK222584 and ZD6474 are being assessed in large size clinical trials (Manley et al 2002). A soluble vascular endothelial growth factor receptor 1 (VEGFR1) chimeric protein has been shown to strongly suppress proliferation of RA endothelial cells *invitro* (Sekimoto et al., 2002). Other promising agents are monoclonal anti-VEGF antibodies which have demonstrated broad spectrum anti-tumour activity, are well-tolerated but may be associated with vascular toxicity such as haemorrhage and thromboembolic

events (Jayson et al., 2002). They are being considered for treatment of non-malignant conditions like rheumatoid arthritis, however in this situation it is worth considering careful monitoring for adverse side effects that may arise from anti-angiogenesis therapy such as: reduced wound healing, infertility and teratogenesis (Walsh 1999).

1.10.8 Therapies from research and development: Gene Therapy

The drugs discussed in the early part of this section 1.10.1 to 1.10.4 can alleviate some of the symptoms associated with arthritis, but cannot stop disease progression. The therapies discussed in the later part of this section 1.10.5 to 1.10.6 have been shown to have a great deal of efficacy. However, this is at the cost of systemic suppression of the whole immune system and reduced wound healing capabilities. All of the therapies discussed so far result in a resurgence of the disease upon cessation of treatment. Therefore, gene therapy has been considered for rheumatoid arthritis as a way of dealing with some or all of these problems.

The concept of gene therapy has been around for a considerable time. The need for drastic therapy in the aggressive treatment of life threatening diseases like cancer has resulted in a rapid progression of this field. However, RA is not considered life threatening and this places constraints upon the types of therapy that can be considered and the type of clinical trials that can be developed. Safety is all important.

Two types of gene therapy exist:

- 1.) In vivo – Genes are delivered directly to the patient, either to the local disease site or systemically.

- 2.) *Ex vivo* – Cells are genetically manipulated in culture and then delivered to the patient locally or systemically.

Naked DNA can be used in gene therapy but it is an inefficient method because it is easily degraded and its uptake by cells is low. Therefore a number of methods for gene delivery have been developed.

Viral vectors from retroviruses and adenoviruses are one of the most commonly employed vehicles. Retroviral-based vectors integrate the therapeutic gene into the host DNA. However, they only infect dividing cells and are therefore used in *ex vivo* methods (Evans et al., 2000).

Adenoviruses infect non-dividing and dividing cells and have been used in direct delivery of therapeutic genes by, intraarticular injection. The results showed efficient, but transient expression of genes. This was mainly due to an immune response generated against the virally-infected cells. (Roessler et al., 1993, Nita et al., 1996, Ghivizzani et al., 1998). This has led to the development of adeno-associated virus vectors these have the genes coding for proteins that induce an immune response deleted. They have been used with some success in the treatment of animal models of arthritis (Pan et al., 2000).

Some non-viral gene transfer methods have also been used, for example liposomes and DNA conjugates were used for local delivery. However, these have demonstrated a low efficiency compared to viral vectors and most tested were found to be pro-inflammatory (Ghivizzani et al., 2001).

There is a wide variety of functions for the therapeutic genes carried by these vectors and they are currently being evaluated for use in the treatment of rheumatoid

arthritis; the most recent being the modulation of pro-inflammatory cytokines. The IL-1 receptor antagonist (IL-1Ra) is a natural receptor antagonist that competes with IL-1 for binding to type I IL-1 receptors and as a result blocks the effects of IL-1 (Arend et al., 1998). Transfer of the IL-1Ra gene into inflamed joints has been evaluated in several animal models. In rats with streptococcal cell wall arthritis, erosion of cartilage and subchondral bone was reduced significantly in joints expressing the transgene (Makarov et al., 1996). In rabbits with antigen-induced arthritis, the results showed reduced inflammation, nearly normalized cartilage matrix destruction and new matrix synthesis (Otani et al., 1996). The first double-blind trials of IL-1Ra gene therapy in humans have been carried out using *ex vivo* gene delivery. The entry criteria for patients into the trial required them to have advanced RA which would necessitate the surgical replacement of the 2nd–5th metacarpophalangeal (MCP) joints on one hand and at least one other joint. Autologous synovial tissue was collected from which synovial fibroblasts were grown. Half of the cells cultured were transduced with an IL-1Ra gene, while the other half was used as controls. The cells were injected intra-articularly into the MCP joints. After 1 week, the MCP joints were surgically replaced and the retrieved tissues examined for evidence of transgene expression. The results showed clusters of cells expressing high levels of IL-1Ra on the synovial surfaces of the relevant joints and no adverse side effects related to gene transfer were reported (Evans et al., 2000). Other genes considered for gene therapy include: TNF receptors, TGF- β , p40 IL-12, soluble CD35 and IL-10 (Robbins et al., 2003). Also, blocking the transcription factor NF- κ B (Tomita et al., 1999) and the ‘genetic synovectomy’ of the proliferating synovium by the delivery of genes that induce the apoptosis of cells within the synovium such as: FasL, TRAIL, and p53 (Robbins et al.,

2003). Recently, extracellular superoxide dismutase (EC-SOD) and catalase genes were transfected into immortalized synoviocytes from Wistar rats. These transformed cells were then grafted into the knee joints of animals induced with monoarticular antigen induced arthritis where they demonstrated over expression of EC-SOD and catalase and lead to amelioration of the disease (Dai et al., 2003). This demonstrates the potential therapeutic benefits in the treatment of rheumatoid arthritis to be gained from genetically enhanced antioxidant enzyme activity.

1.10.9 Therapies from research and development: The final comment

This section has given an indication of the wide range of therapies being developed for the treatment of rheumatoid arthritis. Some of them are not without their controversies, one such being the proposed use of stem cells as a means for tissue repair in arthritic disease (Jorgensen et al., 2001).

All of them demonstrate the need for appropriate animal models for the screening of therapeutic modalities. This subject will be discussed in the final section of my introduction.

1.11. Animal models in the study of rheumatoid arthritis

This introduction has demonstrated that rheumatoid arthritis is a complex disease. The knowledge we have so far, has been gained from patient studies, observations in vitro, and animal models. Historically, animal models have been widely used in medical research. They offer a number of advantages to the study of a disease state:

- 1.) They are genetically controlled thus facilitating the study of genetic links in the aetiology of a disease and also enabling the study of gene mutations to help understand the role of specific genes in pathogenesis.
- 2.) The animal environment can be controlled enabling the continuous monitoring of disease progression without the influence of external factors.
- 3.) Finally, it has been deemed by certain groups of people morally and ethically acceptable to carry out procedures on animals that would not be considered acceptable to be carried out on humans.

Animal models have been shown to mirror much of the pathology of RA and are useful in the investigating the aetiology, pathology and efficacy of a therapy in rheumatoid arthritis (RA). However, there are limitations as no one model can reveal the all the intricacies of a human disease. Therefore, in the study of rheumatoid arthritis several models have been developed each one useful for studying particular aspects of a disease. I have only used one model, adjuvant arthritis, in this thesis. Therefore this one is discussed in depth and other available models discussed briefly.

1.11.1 Detection of hypoxia in the adjuvant arthritis model

Adjuvant arthritis (AA) was the first animal model to be studied for rheumatoid arthritis. It was first induced in rats by Steork (1954) and later developed by Pearson in 1956. It is induced by a single intradermal footpad or tail-base injection of the cell walls of mycobacteria or their components suspended in paraffin oil, so called complete Freund's adjuvant (CFA). 10-14 days after the injection of adjuvant, swelling, warmth and erythema appear in the distal joints of affected animals. Maximum severity of the disease can be

observed between day 14 and 28 post-induction with inflammatory changes observed such as hyperplasia and hypertrophy of the lining synoviocytes in the joints (Pelegrí et al., 1995). In the subintimal lining layer of synovium, the cell density is high and there are a large number of irregularly arranged collagenous fibres. In the joint space next to the synovial lining mononuclear cells and some neutrophils are seen. Pannus formation and bone erosions are also predominant features (Pearson et al., 1961; Stoerk et al., 1961). Subcutaneous injection of CFA also induces a granulomatous inflammation in other organs: spleen, skin, liver, eyes (Pearson et al., 1961). The disease is chronic; however, unlike RA it is self-limiting as the rats recover within 1-2 months. It has been suggested that this is due to the eventual activation of macrophages and the phagocytosis of the CFA (Firestein et al 2000). AA is restricted to rodents and the occurrence of arthritis is dependent on genetic susceptibility. Lewis, Wistar and Sprague-Dawley rats are most commonly used with others, such as Buffalo, being resistant to AA (Bersani-Amado et al., 1990). This susceptibility is related to a gene closely linked to the rat major histocompatibility complex (Battisto et al., 1982).

The aetiology of adjuvant arthritis rather like rheumatoid arthritis is not fully understood, since it requires not only the mycobacteria but also the mineral oil for a response to be generated, in fact, some models use avidine and pristine which contain no cell walls to induce an inflammation that is limited to the joints (Chang et al., 1980, Vingsbo et al., 1995). Heat-shock proteins (HSP) have been shown to play an important role in both AA and RA (de Graeff-Meeder et al., 1990; Ragno et al., 1995) Studies have shown that intra-articular injection of recombinant HSP and a purified protein derivative of tuberculin (PPD), in rats immunised with *Mycobacterium tuberculosis*, induced a chronic

persistent synovitis (Ragno et al., 1995). However, Lewis rats were also protected from developing AA when injected with recombinant mycobacterial 65-kDa hsp (Lopez-Guerrero et al., 1994; Ragno et al., 1997). AA was considered to be a T-cell driven disease, since elimination of T-cells receptor binding was shown to suppress adjuvant arthritis (Yoshino et al., 1990), however, the antigen-specific T cell has not yet been identified (Billingham, 1995). With regard to hypoxia in adjuvant arthritis, as previously mentioned in section 1.4 Bruce (1992) used electron paramagnetic resonance oximetry to measure the partial pressure of oxygen *in vivo* in joints of the adjuvant arthritis model and found a significant decrease in pO_2 between day seven and fourteen post adjuvant arthritis induction.

1.11.2 Other animal models

In the 1980's Sedgwick and co-workers developed a rat model of prolonged immunological inflammation with structural resemblance to synovial changes seen in rheumatoid arthritis. This model was generated by producing a subcutaneous air-filled cavity in the skin of rats previously sensitized to pertussis vaccine. Pertussis vaccine was then injected into 6-day old air pouches (cavities) giving rise to an inflammatory response which lasted up to thirty days. Histological examination of pouch wall tissue showed an early polymorphonuclear infiltration followed by the formation of a lining consisting of mononuclear cells lymphocytes and plasma cells (Sedgwick et al., 1985a). Pertussis was replaced by carrageenan in the formation inflammatory air pouches and this model was used to examine the degradation of articular cartilage *in vivo*. This work indicated that

perhaps the role of inflammatory products was not as significant as other environmental conditions in effecting cartilage destruction in human arthritis (Sedgwick et al., 1985b).

Claxson and co workers (1999) used high field proton NMR spectroscopy to investigate the metabolic status of rat air pouch exudate after induction of inflammation with carrageenan. They demonstrated substantially elevated levels of lactate, low glucose levels and high levels of the ketone body 3-d-hydroxybutyrate, data consistent with a hypoxic environment and consequent anaerobic metabolism. This reflects reported studies of RA synovial fluid and sera that also showed increased levels of lactate, ketone bodies and decreased concentrations of glucose using nuclear magnetic resonance analysis (Naughton et al., 1993a,b). Also as previously mentioned, Soo (1999) used 7-(4'-(2-nitroimidazole-1-yl)-butyl)-theophylline (NITP) to successfully label hypoxic cells in the rat air pouch pseudosynovial tissue. More recently this model has been used to demonstrate activated nuclear factor kappa B (NF κ B) in the intimal and subintimal regions of the air pouch but not in the blood vessels (Ellis et al., 2000). These results contrast the situation in RA where NF κ B has been localised in the vascular endothelium and type 'A' synovial cells from RA patients (Marok et al., 1996).

As well as adjuvant arthritis, Freund's complete adjuvant is used in another model of chronic inflammation, Freund's adjuvant foot pad oedema model. In this model the animals are sensitized with FCA then injected with FCA in the foot pad six days later. Foot pad oedema develops in 24 hours with an intense acute inflammatory response followed by necrosis which intensifies over the next 48 hours.

Another model, monoarticular arthritis, entails pre-sensitization with antigen followed by single joint challenge with antigen. In this case the injected antigen is a

foreign protein like ovalbumin or methylated bovine serum albumin. A chronic inflammatory response develops with bone and cartilage erosions in the challenged joint (Hang et al., 1982).

In recent years another model collagen-induced arthritis (CIA) has become increasingly utilized in the study of rheumatoid arthritis. Immunization is with homologous collagen found in cartilage (type II collagen) and leads to an autoimmune response and severe arthritis (Trentham et al., 1977). Study of this model has revealed a great deal of information about the role of major histocompatibility complex class II molecules and T cells in arthritis and auto-immune responses (Firestein et al., 2000, Morgan et al., 2003). This model has also been used for the screening of target cytokines (Williams et al., 1992, Finnegan et al., 2003) and angiogenesis inhibitors (Peacock et al., 1992).

Adjuvant arthritis is the model to be used in my study because historically it has been used in previous studies of hypoxia and it presents the disease aspects of interest namely: inflammation, synovial hyperplasia, pannus formation and erosion of bone and cartilage.

1.12 Study Hypothesis

This introduction has shown that global assessments of hypoxia, which have been undertaken in the diseased joint, do not reveal specific sites of tissue hypoxia. My hypothesis is that hypoxic synovial cells produce pro-inflammatory cytokines and provide a potential target for bio-reductive therapy and other novel therapies.

The aims of my studies are to use the adjuvant arthritis model to:

1. Identify at what stage during disease progression the joint becomes hypoxic.
2. To examine the phenotype of hypoxic cells.
3. To identify cytokines and transcription factors produced.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials and methods for animal model

2.1.1. Animals

Female Lewis rats (Charles River UK Ltd., Kent) weighing 130-150 g were used for induction of adjuvant arthritis model.

2.1.2. Materials for induction of Adjuvant Arthritis in the animal model

Liquid paraffin - Thornton & Ross Ltd., Huddersfield, UK

Mycobacterium tuberculosis, freeze dried human strains C, DT and PN mixed - Central Veterinary Laboratories, Weybridge, Surrey, UK

Pestle and mortar – Mason & Cook, Stoke-on-Trent, UK.

Sterile syringes & needles - Becton Dickinson, UK

2.1.3 Materials for labelling of hypoxic cells in animal models using Hypoxyprobe

Hypoxyprobe – Chemicon International.

8% Sterile Saline - Sigma, Poole, UK

Sterile syringes & needles - Becton Dickinson, UK

2.1.4 Animal husbandry

The rats were housed in groups of 5 to a cage and exposed to a 12 hour light/dark cycle and an average temperature of $20 \pm 2^{\circ}\text{C}$. Sawdust was used as bedding. All the animals were allowed access to tap water and a standard rat diet (SDS Ltd., Witham, UK) *ad libitum*. The animals were allowed to rest and acclimatize to their new surroundings for at least a week before commencement of experiment.

2.1.5 Induction of Adjuvant Arthritis

Adjuvant arthritis was induced in female Lewis rats using the method of Andrews et al., (1987a, b). A single intradermal injection of a 0.1 ml aliquot of a 10 mg/ml suspension of freeze dried *Mycobacterium tuberculosis* in liquid paraffin oil was administered in the base of the tail of each rat. The suspension of the *M. tuberculosis* was prepared by finely grinding the bacteria in paraffin oil using a pestle and mortar. A small particle size is essential for successful disease induction (Newbould, 1963.)

2.1.6 Clinical assessment

Daily weights were monitored and clinical scores were made on the hind feet according to the following criteria:

Score Value

- | | | |
|---|---|--|
| 0 | - | no inflammation |
| 1 | - | slight redness and swelling of foot, tendons visible. |
| 2 | - | redness, further swelling such that tendons not visible |
| 3 | - | swelling of whole foot including ankle joint. |
| 4 | - | gross swelling of the foot and deformity of ankle joint. |

(Andrews et al, 1987a, b)

2.1.7 Labelling of hypoxic cells using Hypoxyprobe

A single intraperitoneal injection of a 1 ml aliquot of a 60mg/Kg body weight Hypoxyprobe diluted in 0.8% sterile saline was administered to each rat. After termination of the animal model the hypoxic cells were visualized in tissue sections using immunohistochemical techniques.

2.1.8 Termination of the animal model

Animals were killed by cervical dislocation.

2.2 Materials and methods for histological studies

2.2.1 Materials for tissue processing

2-Methylbutane (isopentane) - BDH, Poole, UK

Cork base mold – RA Lamb Ltd., East Sussex, UK

Fibrowax - BDH, Poole, UK

Formic Acid - Sigma, Poole, UK

Histocentre2 – Shandon Inc, Pittsburgh, Pennsylvania, USA

Hypercentre2 – Shandon Inc, Pittsburgh, Pennsylvania, USA

Histological Formal Saline - BDH, Poole, UK

Industrial methylated spirits (IMS) – BDH, Poole, UK

Liquid nitrogen – BOC Ltd, Manchester UK

Tissue-Tek - RA Lamb Ltd., East Sussex, UK

Xylene - BDH, Poole, UK

2.2.2 X-ray materials

Film- Kodak X-ray Film – K. J. Peling, Bristol, UK.

High Fixer – Champion Photochemistry – K. J. Peling, Bristol, UK.

Universal developer - Champion Photochemistry - K. J. Peling, Bristol, UK.

X-ray machine - Faxitron X-ray Corporation, Illinois, USA.

2.2.3 Materials for histological assessment

Acetone - BDH, Poole, UK

Aquamount - BDH, Poole, UK

Background reducing antibody diluent - DAKO, Ely, UK

Concentrated Hydrochloric acid - BDH, Poole, UK

Coverslips - BDH, Poole, UK

DPX mounting media - BDH, Poole, UK

Eosin - Sigma, Poole, UK

Fast Red tablets - Sigma, Poole, UK

Harris's Haematoxylin - BDH, Poole, UK

Industrial methylated spirits (IMS) – BDH, Poole,UK

Mayers Haematoxylin - Sigma, Poole, UK

Normal goat serum - Sigma, Poole, UK

Normal Horse serum - Sigma, Poole, UK

Normal Rabbit serum - Sigma, Poole, UK

Phosphate Buffered Saline (PBS) - BDH, Poole,UK

Pronase antigen retrieval solution - DAKO, Ely, UK

Superfrost microscope slides – BDH, Poole, UK

Tris (hydroxymethyl) methylamine/HCl Buffer (1.5 M, pH 8.8) – Promega, Southampton, UK

Wet-boxes (sealed Perspex containers containing a 10ml water for the purpose of maintaining humidity) – Constructed by University of Bath Engineering Workshop

Xylene - BDH, Poole, UK

2.2.4 Antibodies for immunohistochemical staining

Alkaline phosphatase, Mouse, Vectastain ABC kit – Vector Laboratories Ltd., Peterborough, UK

Alkaline phosphatase, Goat, Vectastain ABC kit – Vector Laboratories Ltd., Peterborough, UK

Alkaline phosphatase, Rabbit, Vectastain ABC kit – Vector Laboratories Ltd., Peterborough, UK

Goat Polyclonal Anti VCAM-1 – Santa Cruz Biotechnology, California, USA.

Mouse Monoclonal Anti Hypoxypore – Chemicon International.

Mouse Monoclonal Anti Fibroblast 5B5 clone – DAKO, Ely, UK

Mouse Monoclonal Anti *Aspergillus niger* glucose oxidase – DAKO, Ely, UK

Mouse Monoclonal Anti Rat ED1 – Serotec, Oxford, UK

Mouse Monoclonal Anti Rat ED2 – Serotec, Oxford, UK

Mouse Monoclonal Anti HIF-1 α – Abcam Ltd., Cambridge, UK

Mouse Monoclonal Anti VEGF – Santa Cruz Biotechnology, California, USA.

Rabbit IgG - DAKO, Ely, UK

Rabbit Polyclonal Anti Ets-1 – Santa Cruz Biotechnology, California, USA.

Rabbit Polyclonal Anti iNOS – BD Biosciences, Oxford, UK

Rabbit Polyclonal Anti Mouse Signal Amplification Kit - DAKO, Ely, UK

2.2.5 Tissue processing methods, paraffin embedding

Tissues were fixed for 24 hours in histological formal saline. The joints were decalcified in a solution of 10% formic acid in histological formal saline for two weeks changing the solution every three days. The joints were then x-rayed (see next section) to determine by loss of radio-opacity whether they had been decalcified. The tissues were then processed through various stages of alcohol and xylene using a Hypercentre2 and embedded in fibrowax using a Histocentre2. Blocks were stored at room temperature until sectioning.

2.2.6 Tissue processing methods, snap freezing

Cork base molds were labelled and covered in tissue-tek a brand of medium for embedding tissue which is frozen. Freshly dissected tissue was then placed on cork base mold and covered with more tissue-tek. Cork base mold was then plunged into 2-methylbutane pre-chilled in a dewer of liquid nitrogen until the block solidified. The block is then removed placed in a bag and stored at -70°C until sectioning.

2.2.7 X-ray of joints

The joints were x-rayed to determine by loss of radio-opacity whether they had been fully decalcified. This was necessary to prevent damage to the microtome blade during subsequent histological sectioning. The x-ray film was placed inside a plastic cover and the joints were arranged on top of the piece of plastic. The piece of plastic was then placed in the x-ray cabinet of the Faxitron and the film exposed for 20 minutes at 40 kilo-volts potential (kvp). Inside a dark room, the x-ray film was developed for 3 minutes and fixed

for 2 minutes. The x-ray film was then rinsed in running water before being dried and examined using a light-box.

2.2.8 Haematoxylin and Eosin staining of sections

Sections (5µm) were cut from wax embedded tissue blocks on a sledge microtome and mounted on Superfrost slides. The sections were allowed to air dry and were then baked in an oven at 60°C overnight. Tissue sections were dewaxed in xylene (x2) for 5 min, washed in 100% IMS (x2) for 2 min and immersed in running tap water for 5 min. The previous section was omitted for frozen sections which were cut 10µm thick, mounted on Superfrost slides, air dried for 1 hour fixed with acetone for 5 minutes and rehydrated in tap water before use. All slides were then stained as follows:

- i) Stain in Harris's Haematoxylin solution for 5 min.
- ii) Wash in running tap water for 5 min.
- iii) Differentiate in acid alcohol (1% hydrochloric acid in 100% IMS) for 1 min.
- iv) Wash in running tap water for a further 5 min.
- v) Stain in Eosin solution for 30 sec.
- vi) Wash in running tap water for 5 min.
- vii) Wash in 100% IMS for 2 min. (x2).
- viii) Clear in xylene for 2 min. (x2).
- ix) Finally, the tissue sections were mounted under glass coverslips using DPX mounting media and were dried at room temperature overnight prior to microscopy.

2.2.9 Immunohistochemical staining methods

2.2.9.1 Immunohistochemical staining with antigen retrieval and using alkaline phosphatase kit.

5µm thick sections were cut from wax embedded tissue blocks on a sledge microtome and mounted on Superfrost slides. The sections were allowed to air dry and were then baked in an oven at 60°C overnight. Tissue sections were dewaxed in xylene (x2) for 5 min, washed in 100% IMS (x2) for 2 min and immersed in running tap water for 5 min. This section was omitted for frozen sections which were cut 10µm thick and mounted on Superfrost slides, air dried for 1 hour and fixed with acetone for 5 minutes before use. All slides were then stained as follows:

All procedures carried out at room temperature, unless otherwise stated

- i) Rinse twice in phosphate buffered saline (PBS) for 5 minutes.
- ii) Incubate with a 0.01% solution of Pronase diluted in Tris buffered saline pH 7.2 (TBS) at 40°C for 40 minutes in wet box
- iii) Rinse twice in phosphate buffered saline (PBS) for 5 minutes.
- iv) Incubate with a 1:5 dilution of normal serum from species in which secondary antibody was generated for 20 min in a wet box.
- v) Tap off serum and incubate with the appropriate antibodies overnight at 4°C in a wet box.
- vi) as in step (iv)
- vii) Incubate with a 1:200 dilution of secondary antibody from an alkaline phosphatase, vectastain ABC kit for 30 min in a wet box.

- viii) as in step (iv)
- ix) Incubate with a 1:100 dilution of avidin-alkaline phosphatase complex from an alkaline phosphatase, vectastain ABC from the kit for a further 30 min in a wet box.
- x) as in step (iv)
- xi) Develop to reveal phosphatase (red reaction product) using fast red tablets. Stop reaction using tap water when the tissue sections have been sufficiently developed.
- xii) Lightly counterstain with Mayers haematoxylin for 1 min, then rinse in tap water.
- xiii) The tissues are mounted under glass coverslips using aqua mounting media and left to dry overnight at room temperature prior to microscopy.

2.2.9.2 Immunohistochemical staining using alkaline phosphatase kit.

Sections (5µm) were cut from wax embedded tissue blocks on a sledge microtome and mounted on Superfrost slides. The sections were allowed to air dry and were then baked in an oven at 60°C overnight. Tissue sections were dewaxed in xylene (x2) for 5 min, washed in 100% IMS (x2) for 2 min and immersed in running tap water for 5 min. This section was omitted for frozen sections which were cut 10µm thick and mounted on Superfrost slides, air dried for 1 hour and fixed with acetone for 5 minutes before use. All slides were then stained as follows:

All procedures carried out at room temperature, unless otherwise stated

- (i) Rinse twice in phosphate buffered saline (PBS) for 5 minutes.
- (ii) Incubate with a 1:5 dilution of normal serum from species in which secondary antibody was generated for 20 min in a wet box.

- (iii) Tap off serum and incubate with the appropriate antibodies overnight at 4°C in a wet box.
- (iv) Rinse twice in phosphate buffered saline (PBS) for 5 minutes.
- (v) Incubate with a 1:200 dilution of secondary antibody from an alkaline phosphatase, vectastain ABC kit for 30 min in a wet box.
- (vi) As in step (v).
- (vii) Incubate with a 1:100 dilution of avidin-alkaline phosphatase complex from an alkaline phosphatase, vectastain ABC kit for a further 30 min in a wet box.
- (viii) As in step (v).
- (ix) Develop to reveal phosphatase (red reaction product) using fast red tablets. Stop reaction using tap water when the tissue sections have been sufficiently developed.
- (x) Lightly counterstain with Mayers haematoxylin for 1 min, then rinse in tap water.
- (xi) The tissues were mounted under glass coverslips using aqua mounting media and left to dry overnight at room temperature prior to microscopy.

2.2.9.3 Immunohistochemical staining using signal amplification kit.

Sections (5µm) were cut from wax embedded tissue blocks on a sledge microtome and mounted on Superfrost slides. The sections were allowed to air dry and were then baked in an oven at 60°C overnight. Tissue sections were dewaxed in xylene (x2) for 5 min, washed in 100% IMS (x2) for 2 min and immersed in running tap water for 5 min. This section

was omitted for frozen sections which were cut 10µm thick and mounted on Superfrost slides, air dried for 1 hour and fixed with acetone for 5 minutes before use. All slides were then stained as follows:

All procedures carried out at room temperature, unless otherwise stated

- (i) Rinse twice in phosphate buffered saline (PBS) for 5 minutes.
- (ii) Incubate with 3% Hydrogen peroxide solution for 20 min.
- (iii) Rinse twice in phosphate buffered saline (PBS) for 5 minutes.
- (iv) Incubate with protein block from mouse signal amplification kit, for 20 min in a wet box.
- (v) Tap off block and incubate with the appropriate antibodies diluted in background reducing antibody diluent overnight at 4°C in a wet box.
- (vi) As in step (IV).
- (vii) Incubate with secondary antibody from signal amplification, mouse, kit for 15 min in a wet box.
- (viii) As in step (IV).
- (ix) Incubate with strep-biotin complex from mouse signal amplification kit for 15 min in a wet box.
- (x) As in step (IV).
- (xi) Incubate with amplification reagent from mouse signal amplification kit for 15 min in a wet box.
- (xii) As in step (IV).
- (xiii) Incubate with strep-peroxidase complex from mouse signal amplification kit for 15 min in a wet box.

- (xiv) Develop to reveal peroxidase (brown reaction product) using 3,3-diaminobenzidine (DAB) substrate (from kit). Stop reaction using tap water when the tissue sections have been sufficiently developed.
- (xv) Lightly counterstain with Mayers haematoxylin for 1 min, then rinse in tap water.
- (xvi) The tissues were mounted under glass coverslips using aqua mounting media and left to dry overnight at room temperature prior to microscopy.

2.2.10 Histology imaging

Histological sections were examined using a Zeiss Axioscope II (supplied by Zeiss, UK) with a JVC 3-CCD camera for digital imaging (supplied by Imaging Associates Ltd., Thame, Oxford, UK.) 5X, 10X, 20X and 40X microscope objectives were used with white light illumination and digital images stored as JPEG files.

2.2.11 Histological assessment

Changes in the morphology of the joints using Haematoxylin and Eosin (H&E) staining were observed and scored on a scale of 0-4 when compared to naive controls that had a score of zero. The following criteria were used:

| Parameter | Score |
|-----------------------|-------|
| Synovial hyperplasia | 0-4 |
| Cellular infiltration | 0-4 |
| Bone erosion | 0-4 |

(Kruijsen et al, 1983.)

2.2.12 Immunohistochemical assessment

Blind sections of hind feet were observed using a 10X microscope objective. Image analysis was carried out on all areas of joint tissue except skin to determine the percentage of stained area compared to unstained area. The following self-written macro for Zeiss 3.0 was used:

```
tvlive  
  
MSload "x10lenscalibration"  
  
tvframeinput 1,0,0,800,600  
  
dislevrgb 1,2,0,0,0,255,2,204,0,218,10,"RGB"  
  
binscrap 2,2,10,100000000,1  
  
MSsetframe  
  
MSsetfeat "FIELDFEAT"  
  
! MSmeasmask 2,1,"caren3",0,2,10  
  
imgdelete 2  
  
imgdelete 1  
  
tvlive
```

This program analyses a framed area of 800 by 600 pixels. It removes from the analysis any area less than $10\mu\text{m}^2$, thus removing small histological artefacts. Large histological artefacts, for example, areas of folded tissue were manually removed from the analysis by outlining them. Hypoxic regions were thresholded based upon a pimonidazole adduct density converted to a red-blue-green level which was consistent throughout the

images. Since spatial transition between peak density and 0% (non hypoxic area) was quite distinct this provided a reproducible method to demarcate hypoxic areas. Finally the program calculates the demarcated area as a percentage of the whole framed area. These data were then tabulated using Graph Pad Prism software for statistical analysis.

2.3 Statistics

Comparisons of data for naïve and adjuvant arthritis joints were assessed using the Mann Whitney test.

2.4 Materials and methods for tissue explant and cell culture

3.4.1 Cell and tissue culture materials

Accutase- Innovative Cell Technologies, Inc, California, USA.

Antibiotic antimycotic, Penicillin/Streptomycin – Invitrogen, Paisley, UK

Dimethyl sulfoxide (DMSO) - Sigma, Poole, UK

Dulbecco's modified Eagle's medium - Invitrogen, Paisley, UK

Dulbecco's phosphate buffered saline (PBS) - Invitrogen, Paisley, UK

Foetal calf serum (FCS) - Invitrogen, Paisley, UK

Hank's balanced salt solution - Invitrogen, Paisley, UK

Sterile T75 Flasks – Triple Red, Oxon, UK

Sterile 6-well plates - Triple Red, Oxon, UK

Sterile pipettes - Triple Red, Oxon, UK

2.4.2 Cell lines and tissue explants.

HeLa from American Type Culture Collection, Manassas, VA, USA.

Human neonatal foreskin fibroblast cells generously supplied by Dr. Vivek Mudera, Deputy Director of Tissue Repair Unit and Tissue Engineering Centre and the Centre for Plastic and Reconstructive surgery at Royal Free University College Medical School, University college London.

Human rheumatoid arthritis synovial fibroblast cells generously supplied by Dr. D. Speden Department of Medical Sciences University of Bath.

Whole patella suspensory ligaments and synovium dissected from naïve and adjuvant female Lewis rats(Charles River UK Ltd., Kent) generously provided by Dr Paul I. Mapp, Department of Medical Sciences, University of Bath.

2.4.3 Defrosting cells and culturing cells

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with a 10% FCS and 1% Penicillin/Streptomycin solution. This will be called complete medium. All procedures in chapter 3.4.3 to 3.4.6 were carried out using aseptic techniques within a level two containment laminar flow cabinet. The cells were defrosted rapidly by placing the vial of cells into a water bath at 37°C, and the contents were pipetted into a sterile 15 ml tube with a 10ml volume of complete medium. The cells were then centrifuged at 1000 rpm before being resuspended in 10 ml of complete medium. The cells were then grown in a T75 flask with complete medium in a humidified incubator at 37°C

and a 5% CO₂ / 95% air mix. The media were changed every 2-3 days and cells were allowed to grow until confluent.

2.4.4 Passaging cells

Confluent cells were either passaged for use in experiments or storage. Cells to be passaged were first washed with Dulbecco's phosphate buffered saline solution (PBS) and then 2ml Accutase was added. The flasks were then incubated at 37°C until the cells became detached (approximately 10 min). Once cells had become detached, an equal volume of complete medium was added to prevent further enzymic action. The cells were centrifuged for 10 min at 1000 rpm and resuspended in 5 mls of DMEM. They were then separated into 1 ml aliquots and either pipetted into T75 flasks for culturing or frozen down for storage.

2.4.5 Freezing down cells

Cells from confluent cultures were frozen down in liquid nitrogen and stored for later use. The cells were passaged as described previously in 3.4.4 and resuspended into 5 mls of complete medium. A 1 ml aliquot of a 10% dimethylsulfoxide (DMSO) in FCS was added to the cryotube followed by a 1 ml aliquot of the cells that had been resuspended in complete medium. The cryotubes were then placed into a freezing container in a -70°C freezer overnight before being stored in liquid nitrogen.

2.4.6 Culturing tissue explants

Whole patella suspensory ligaments and synovium was dissected from naïve and adjuvant female Lewis rats and placed in sterile bijoux containing 4ml of Hanks balanced salt solution with 1% Penicillin/Streptomycin solution. The explants were washed four times for 5 min with this solution before being aseptically transferred to individual wells in a six well plate containing 4ml of complete medium. The explants were then incubated in either the hypoxia cabinet (see 3.5) or a humidified incubator at 37°C and a 5% CO₂ / 95% air mix.

2.5 Materials and method for hypoxia cabinet operation

2.5.1 Materials for hypoxia cabinet operation

Microflow Anaerobic System (hypoxia cabinet) – M.D.H Ltd., Hampshire, UK.

Gas supply cylinder with 0% O₂ / 5% CO₂ / 95%N₂ - BOC Ltd, Manchester UK

Wet-boxes (sealed perspex containers containing 10 ml water for the purpose of maintaining humidity) – Constructed by University of Bath Engineering Workshop

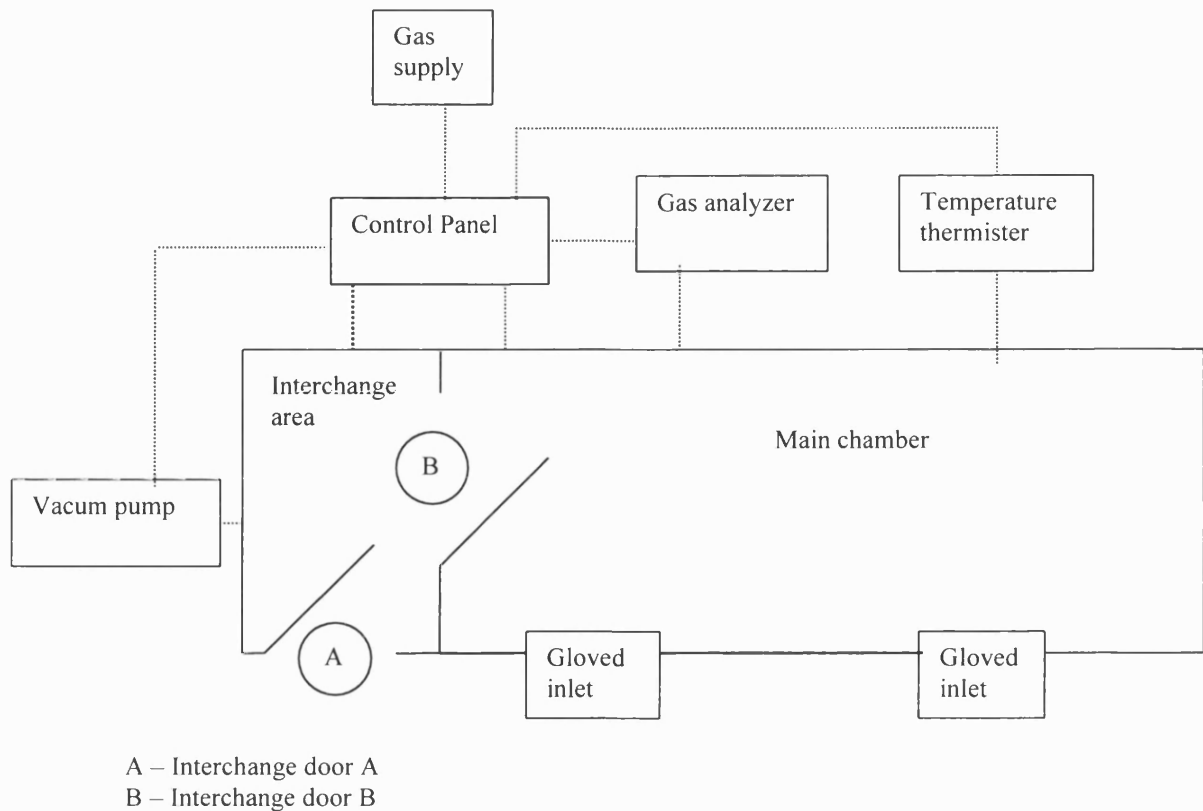


Figure 2. Representative diagram of the top view of a hypoxia cabinet (Microflow Anaerobic System)

2.5.2 Method for hypoxia cabinet operation

- i) Place wet boxes in main chamber (Figure 2). Ensure both interchange doors and gloved inlets are closed and use control panel to set the main chamber environmental conditions, temperature at 37°C and gas supply 5% CO₂ / 0% O₂ / 95% N₂ mix for 24 hrs before experiment commences, to allow cabinet to equilibrate.

- ii) Temperature and oxygen tension can be checked with additional probes placed in the main chamber.
- iii) To commence the experiment open interchange door A and place plates and flasks in the interchange area close the door.
- iv) Use control panel to run a vacuum cycle. This runs a three minute flushing cycle to fill the interchange area with the same environmental conditions set previously for the main chamber in step I). A green light will indicate completion.
- v) Open gloved inlet and places hands in sealed gloves. Open interchange door B and remove plates and flasks place in wet boxes. Close all interchange doors and gloved inlets and incubate for fourteen days under the set environmental conditions.

Steps iii) to v) must be repeated to add or remove any equipment to the main chamber and to terminate the experiment under the set environmental conditions.

2.6 Materials and methods for protein isolation

2.6.1 Materials for protein isolation

Biofuge - Heraeus Instruments, UK

Chloroform - Sigma, Poole, UK

Eppendorfs – Alpha Labs, Hampshire, UK

Ethanol - Sigma, Poole, UK

Guanidine hydrochloride - Sigma, Poole, UK

Isopropyl alcohol - Sigma, Poole, UK

RNAase, Stat60 - AMS Biotechnologies, Oxford, UK

2.6.2 Method for protein isolation

Protein Precipitation

- i) Protein was isolated from the cells by treatment with 2ml RNAase, Stat60, per T75 flask which was then stored in -70°C till use.
- ii) Samples thawed at room temp for 5 min.
- iii) Add 200µl Chloroform per 1ml of protein and Stat 60 mix .
- iv) Cover and shake vigorously for 15 min.
- v) Leave at room temp for 15 min.
- vi) Centrifuge at 12000 rpm for 15 min.
- vii) Transfer aqueous phase to fresh tube and save for RNA isolation.
- viii) Remove and overlaying liquid from remaining tube and add 1.5 ml Isopropyl alcohol used to organic phase.
- ix) Store at room temp for 5 min.

- x) Centrifuge at 12000 rpm for 10 min.

Protein Wash

- xi) Remove supernatant and add 2ml of 0.3M guanidine hydrochloride.
- xii) Store at room temp for 20 min.
- xiii) Centrifuge at 7500 rpm for 5 min
- xiv) Add 2ml of 100% ethanol.
- xv) Vortex.
- xvi) Store at room temperature for 20 min.
- xvii) Centrifuge at 7500 rpm for 5 min.
- xviii) Repeat protein wash steps x) to xvi) three times.

Protein Solubilisation

- xix) Dry pellet at room temp for 10-15 min.
- xx) Dissolve in 10% SDS for up to 16 hours at 50°C.
- xxi) Sonication of solution for 15 min may be required to dissolve protein.
- xxii) Centrifuge at 10000 rpm for 10 min.
- xxiii) Transfer supernatant to fresh tube.
- xxiv) Use for protein assay, western blot or sample may be stored at -70°C.

2.7 Materials and Methods for Protein Assay

2.7.1 Materials for Protein Assay

Biofuge - Heraeus Instruments, UK

DC Protein Assay Kit – BioRad, Herts, UK

96-well Microplates – Triple Red, Oxon, UK

Protein Standard – BioRad, Herts, UK

10% Sodium dodecyl sulphate (SDS) – Promega, Southampton, UK

2.7.2 Method for Protein Assay

- i) Use protein assay kit to prepare ‘working solution’ by adding 20µl of reagent ‘S’ to each ml of reagent ‘A’ required for working.
- ii) Prepare 5 dilutions of a protein standard containing 0.2mg/ml to 1.5mg/ml protein.
- iii) In triplicate pipette 5µl of standards and samples into clean, dry microtiter plate.
- iv) Add 25µl of ‘working solution’ into each well.
- v) Add 200µl of reagent ‘B’ to each well and mix.
- vi) After 15 min read at 750nm and generate standard curve to calculate protein in samples.

2.8 Materials and methods for gel electrophoresis & western blotting

2.8.1 Materials for Gel Electrophoresis & Western Blotting

Acrylamide – Promega, Southampton, UK

Ammonium Persulphate (AMPS) – Sigma, Poole, UK

Antibodies - Mouse Monoclonal Anti HIF-1 α – Novus, Littleton, CO, USA

- Rabbit Polyclonal Anti Ets-1 – Santa Cruz Biotechnology, California, USA.

Automated developer – Fuji, UK

Autoradiography film – Kodak, UK

Biofuge - Heraeus Instruments, UK

Bromophenol blue (0.2%) – Sigma, Poole, UK

Butan-2-ol – BDH, Poole, UK

Chromic acid – BDH, Poole, UK

ECL detection kit – Amersham, UK

Gel electrophoresis tank and peripheral equipment – Jencons Scientific Ltd., Beds, UK

Glycerol (40%) – Sigma, Poole, UK

Non-fat dried milk – Sainsbury's,

Nitrocellulose paper – GRI, UK

Rainbow markers (molecular weights, 250-10kD) – Amersham, UK

SaranWrap™ – BDH, Poole, UK

10% Sodium dodecyl sulphate (SDS) – Promega, Southampton, UK

Tetramethylethylenediamine (TEMED) – Promega, Southampton, UK

Tris (hydroxymethyl) methylamine/HCl Buffer (1.5 M, pH 8.8) – Promega, Southampton, UK

Tris-HCl buffer (250 mM, pH 7.5) - Sigma, Poole UK

Western blotting tank - Jencons Scientific Ltd., Beds, UK

Whatman Paper - Whatman Limited, UK

2.8.2 Preparation of the polyacrylamide gel plates.

Two plates were cleaned by immersing into chromic acid followed by rinsing under running tap water. They were clamped together with a gasket inserted between them to create a space for the gel. A (7.5%) acrylamide running gel was prepared (see appendix A). The solution was then carefully poured in between the plates until it reached 2 cm from the top of the plates. An overlayer of water-saturated butan-2-ol was added to remove the

meniscus. Once the gel had polymerized (approximately 1 hour), it was drained and rinsed with distilled water. The plates were then filled to the top with a (5%) acrylamide stacking gel (see Appendix A). The well combs were inserted and the gel was left to polymerize. Once the gel had set, the combs were removed.

2.8.3 Sample solution Preparation.

An equal volume of 2x reducing sample buffer (see appendix A) was added to each sample containing equal amounts of total protein (10 μ g) in an Eppendorf and boiled for 3 mins. The samples were then centrifuged at 10,000 rpm for 5mins.

2.8.4 Gel Electrophoresis

The gel plates were placed in the gel electrophoresis tank which was filled with electrophoresis buffer pH 8.3 (see appendix A). An equal volume (10-30 μ l) of sample solutions were carefully loaded into the gel wells. The gel was then subjected to electrophoresis at a constant voltage of 100V for approximately 2 hours, or until the tracking dye reached the base of the gel

2.8.5 Western Blot and Immunodetection by ECL.

Following electrophoresis the protein was transferred from the gel to nitrocellulose paper, on which the blots were prepared. This was achieved by the following process (all procedures carried out at room temperature unless otherwise stated):

- i) After electrophoresis carefully transfer the gel to a container of blotting buffer, (see Appendix A) for 10 mins.

- ii) Cut nitrocellulose paper and Whatman paper to the size of the gel and incubate in the blotting buffer for 10mins.
- iii) Use a western blot cassette holder and place 6 filter papers on top of the sponges on both sides of the holder.
- iv) Place the nitrocellulose paper and the gel on one set of filter papers, carefully remove all air bubbles.
- v) Place the second set of filter papers on top of the gel and close the cassette.
- vi) Transfer cassette to a western blotting tank make sure that the nitrocellulose paper is nearest to the positive electrode (anode) as shown in Figure 3

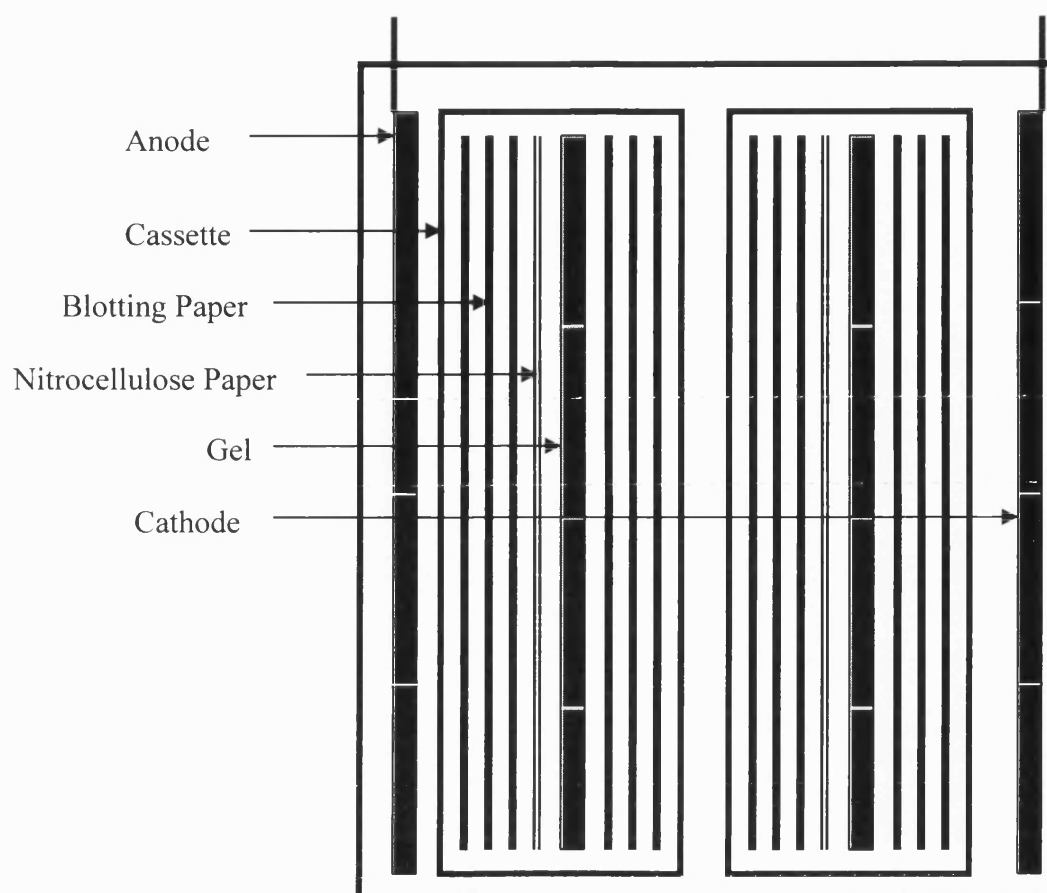


Figure 3. Western blotting tank setup

- vii) Add blotting buffer to the tank and run protein blot at 150mA for 2 hours.
- viii) Remove cassette from the tank.
- ix) Stain gel with Coomassie Brilliant Blue (see Appendix A) to check for complete transfer of protein to the nitrocellulose paper.
- x) Mark the positions of different molecular weight markers on the nitrocellulose paper with pencil.
- xi) Incubate the nitrocellulose paper in blocking buffer consisting of 5% non-fat dried milk in 0.5% Tween 20/1 x PBS overnight at +4°C.
- xii) Wash once with 0.5% Tween 20/1 x PBS for 5 min.

- xiii) Incubate in a square petri dish on a rocking plate with 5mls of diluted primary antibody in 5% non-fat dried milk in 0.5% Tween 20/1 x PBS for 2 hours.
- xiv) Wash 3x in 0.5% Tween 20/1 x PBS 3 times, for 10 min each.
- xv) Incubate with diluted secondary antibody 1:2500 dilution in 5% non-fat dried milk in 0.5% Tween 20/1x PBS) for 1 hour.
- xvi) Wash as in step xiv).
- xvii) Mix equal volumes of solution A and solution B from the ECL Amersham detection kit and add to the nitrocellulose paper making sure it is completely covered. Incubated for 2 min.
- xviii) Drain excess drain off excess liquid and cover in SaranWrap. Smooth out all bubbles and placed in a film cassette, protein side up.
- xix) In a dark room, place a sheet of Kodak autoradiography film on top of the membrane and expose for 30secs.
- xx) Develop film using an automated developer.
- xxi) Further films can be exposed and developed, up to 12 hours to ensure resolution of all details.

2.8.6 Assessment of western blots

Blots were analyzed by scanning densitometry (Scion Image Beta 4.02 - Scion Corporation, Maryland, USA.) Data plotted in Excel.

CHAPTER 3: Determining the Optimum Time Point for Administering the Hypoxyprobe

3.1 Introduction

Hypoxyprobe-1 is a commercially available injectable preparation of pimonidazole (a modified 2-nitromidazole). It is taken up by all tissues in the body but binds only to cells with a pO_2 of 10 mm Hg (or less) at 37°C. Hypoxyprobe-1MAB1 is a monoclonal antibody that binds to protein adducts of Hypoxyprobe produced exclusively in hypoxic cells and this allows their detection in tissues *ex-vivo*. At the time that this study was carried out pimonidazole had only been used to measure low oxygen in animal and human tumours as an index of radiation resistance (Kim et al., 1993, Kennedy et al., 1997). As this study uses the adjuvant arthritis model in Lewis rats a pilot study was carried out to determine the optimum time point (in terms of labeling efficacy) for administering the hypoxyprobe prior to killing the animal. I felt that this initial study to establish labeling efficacy with pimonidazole was essential before the more complex time-course studies were undertaken. Previous studies from the department using oximetry have shown that the adjuvant joint is most hypoxic between days 7 and 14 post adjuvant induction when clinical disease was well developed (Bruce, 1992). Day 14 was therefore used as maximal hypoxia and maximal histological change were likely to be present.

3.2 Materials and Methods

Adjuvant arthritis was induced in 4 groups of female Lewis rats (n=4) as described in part 2.1.5. Also there was one group of naïve control rats (n=4). Rats from each group were killed on Day 14. Hypoxyprobe was injected intraperitoneally as described in chapter 2.1.7 at 30 min, 2 hours, 6 hours or 18 hours before the rats were killed, to establish the

optimal binding time for the hypoxyprom. One group was left without hypoxyprom. The rats were killed by cervical dislocation and their hind feet were then removed and used for subsequent histological investigations as described in chapter 2.2. Hypoxyprom-1 adducts were detected using immunohistological method described in 2.2.9.1.

3.3 Results

Three types of negative controls for the mouse anti hypoxypore detection procedure were carried out:

- 1.) Sections from adjuvant arthritis hind feet from animals not injected with hypoxypore.
- 2.) Sections from adjuvant arthritis hind feet from hypoxypore injected animals but without the primary antibody, mouse anti-hypoxypore.
- 3.) Sections from adjuvant arthritis hind feet with hypoxypore using an irrelevant primary antibody, mouse anti-*Aspergillus niger* glucose oxidase

All negative controls were clear of staining and showing a lack of antibody cross-reactivity and non-specific staining (plates 3.1, 3.2 and 3.3.) Rats injected with hypoxypore 30 minutes and 2 hours prior to termination were found to have a high level of staining for hypoxypore within the plasma (plates 3.4 and 3.5.) This indicates that the hypoxypore had not been adequately cleared from the circulation. Staining within the 6-hour and 18 hour injected animals had plasma clear of unbound hypoxypore and distinct labelling of cells bound to hypoxypore (plates 3.6 and 3.7.) All labelled adjuvant hind feet demonstrated the following characteristic staining:

Bone marrow staining for hypoxypore and chondrocyte staining on the surface of the cartilage near the invading pannus. Also staining of distinct clusters of cells within the inflammatory infiltrate was seen.

All naïve 6 hour and 18 hour control rats showed very little staining; there was a discrete pale stain in some chondrocytes (plate 3.8.)

3.3 Results

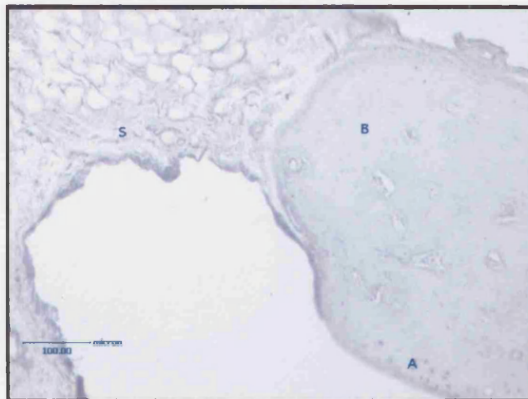


Plate 3.1 Negative control: Adjuvant hind foot joint, without hypoxypore (x10,wax section, stained using mouse anti-hypoxypore.) Articular Cartilage [A]; bone [B] and synovium [S].

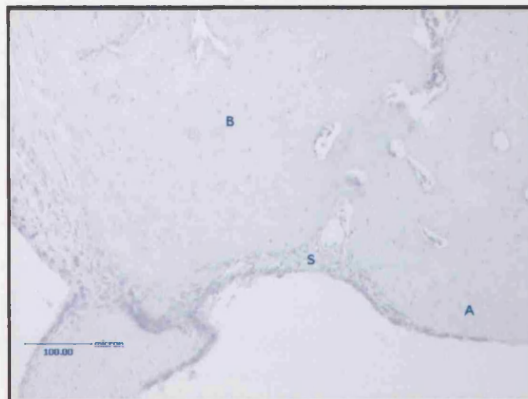


Plate 3.2 Negative control: Adjuvant hind foot joint, with hypoxypore (x10,wax section, stained without using mouse anti-hypoxypore.) Articular Cartilage [A]; bone [B] and synovium [S].

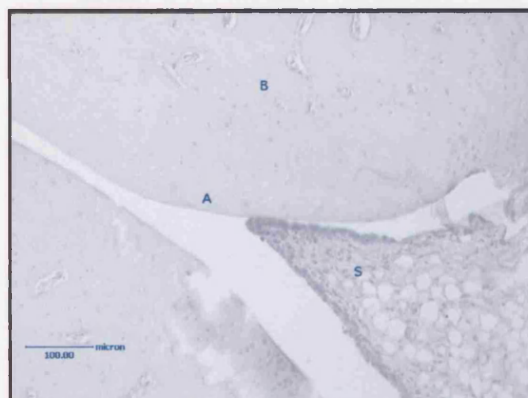


Plate 3.3 Negative control: Adjuvant hind foot joint, with hypoxypore (x10,wax section, stained using irrelevant mouse antibody.) Articular Cartilage [A]; bone [B] and synovium [S].

Plate 3.4 30 min. hypoxyprobe: Adjuvant hind foot joint (x10 wax section, stained with mouse anti-hypoxyprobe.) Blood vessels[BV], bone [B] and synovium [S]. Note high background stain and unbound hypoxyprobe (red reaction product) in the blood vessel lumen [arrows].

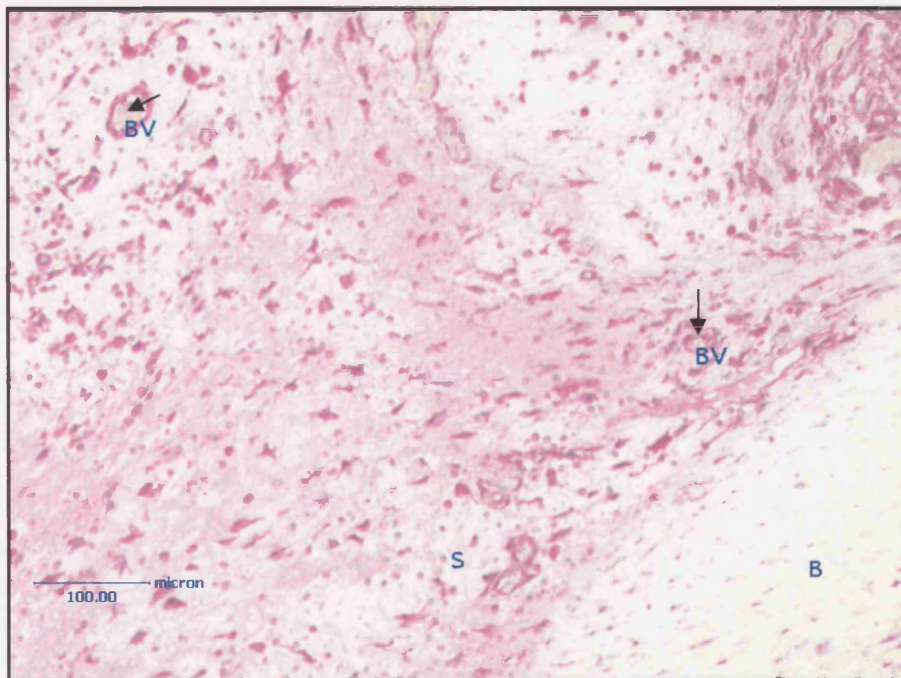


Plate 3.5 2 hour hypoxyprobe: Adjuvant hind foot joint (x10 wax section, stained with mouse anti-hypoxyprobe.) Blood vessels[BV], bone [B] and synovium [S]. Note unbound hypoxyprobe in the blood vessel lumen [arrows].

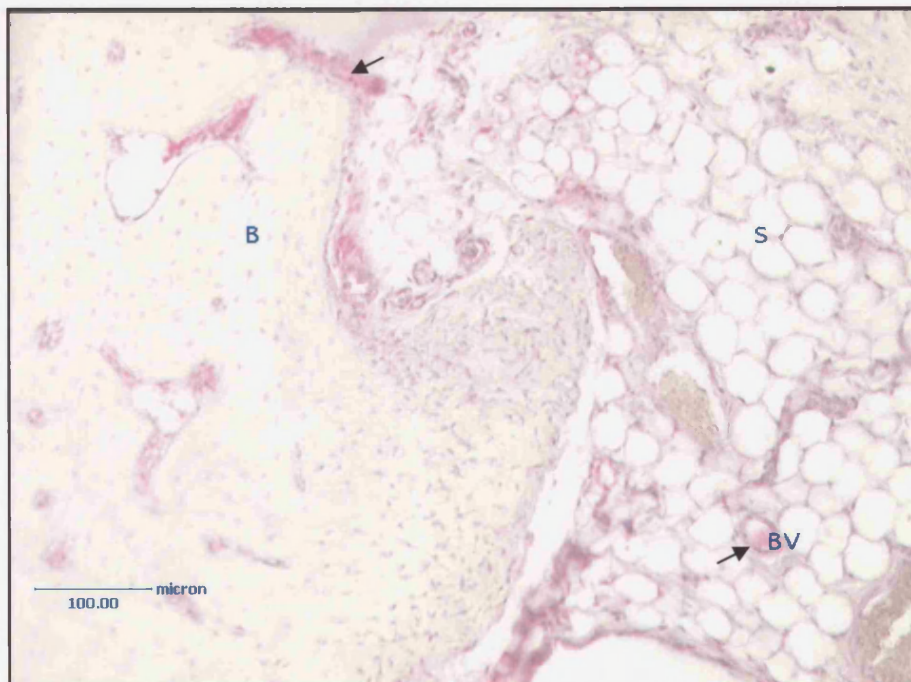


Plate 3.6 6 hour hypoxyprobe: Adjuvant hind foot joint (x10 wax section, stained with mouse anti-hypoxyprobe.) Blood vessels[BV], bone [B] and synovium [S]. Note clear specific extra-vascular staining [arrows] and blood vessels clear of unbound hypoxyprobe.

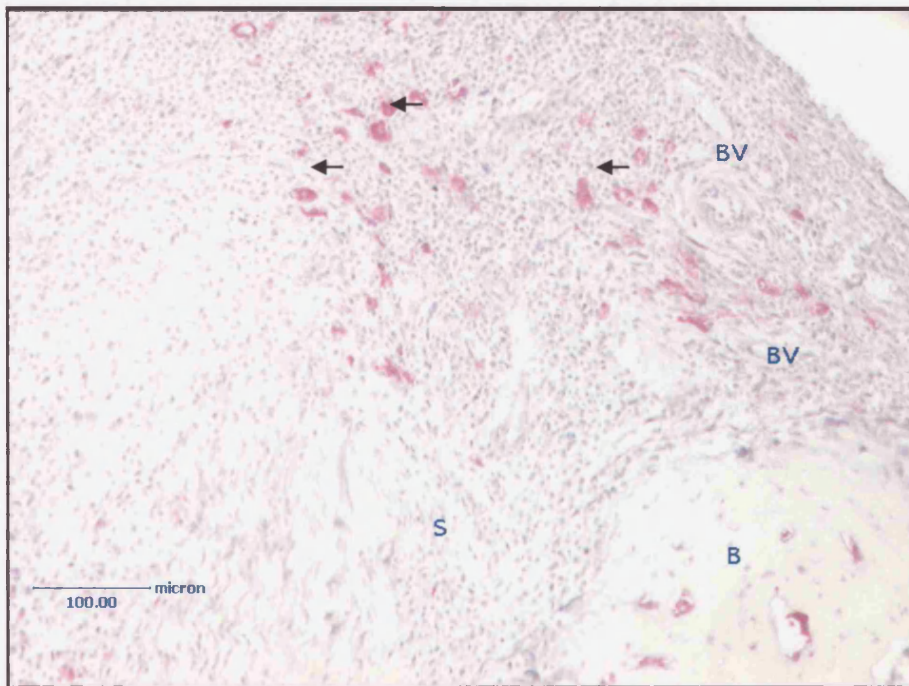


Plate 3.7 18 hour hypoxyprobe: Adjuvant hind foot joint (x10 wax section, stained with mouse anti-hypoxyprobe.) Bone [B], articular cartilage [C] and synovium [S]. Note specific staining [arrows].

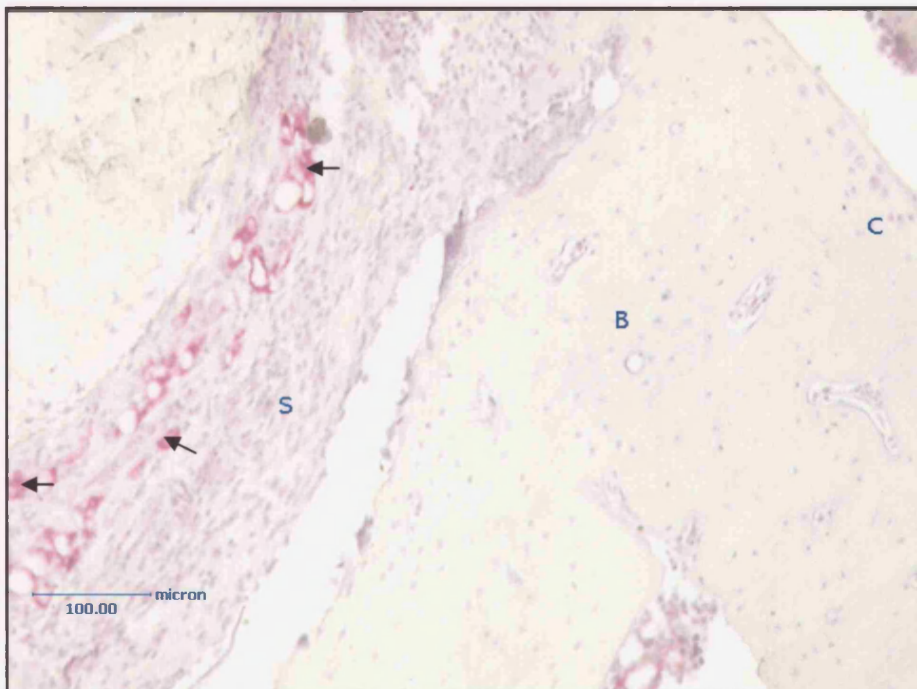
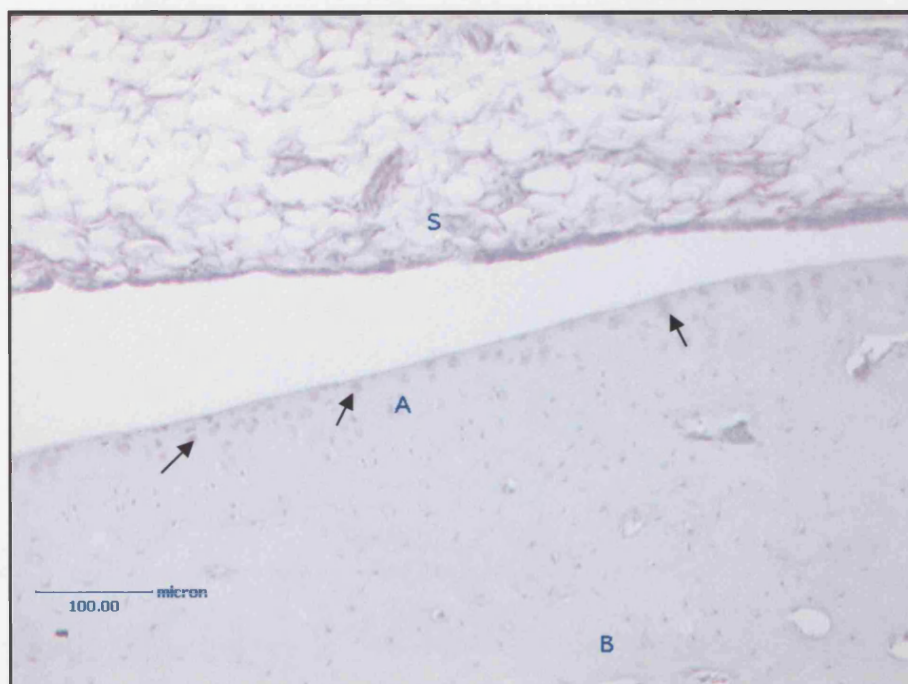


Plate 3.8 6 hour hypoxyprobe: Naive hind foot joint (x10 wax section, stained with mouse anti-hypoxyprobe.) Articular cartilage[A], bone [B] and synovium [S]. Note minimal staining of chondrocytes [arrows].



3.4 Discussion.

Based on this preliminary study it was decided that a large study would be designed to establish at what point during the disease course of adjuvant arthritis the tissue of the inflamed joint became hypoxic. This study would also be used to quantify the difference between the staining in the adjuvant rat joint and the staining seen in the naïve controls. Finally based upon the evidence from the study (3.1), the 6-hour time point for dosage of the animals prior to termination was chosen because it was the shortest time that gave clear staining for hypoxypromide adducts. It was decided that the 18-hour time point was unsuitable despite the clear staining for hypoxypromide adducts this gave. The reason for this is based on the speed of disease progression in the adjuvant rat. An animal with a clinical score of 1 dosed with hypoxypromide may well have a higher clinical score 18 hours later when it is killed.

CHAPTER 4: Characterisation of the Time Course of Hypoxia in the Synovial Joint of Adjuvant Arthritis

4.1 Introduction

The objective of this study was to quantitatively compare the level of hypoxia in the adjuvant arthritis inflamed joint to the naive matched controls and establish at what point during the disease progression the joint becomes hypoxic.

4.2 Materials and Methods

60 Female Lewis Rats were caged in groups of five. Adjuvant arthritis was induced in thirty rats (n=30) as described in part 2.1.5. The remaining rats (n=30) were left as non-diseased time matched controls. The disease progression was monitored by measuring body weight daily and clinically scoring the hind limbs according to the method of Andrews et al. (1987a,b). The method is fully described in section 2.1.6.

Rats were killed at time points and clinical scores shown in Table 1. Number of animals available with a particular clinical score on a specific day is dependent on disease progression in the groups.

| Time Point | Clinical Score | Number of Hind Feet Collected |
|--|-----------------------|--------------------------------------|
| Day 0 pre adjuvant arthritis (AA) induction | 0 | 5 |
| Time matched naïve controls day 0 pre AA induction. | 0 | 5 |
| Day 3 post-AA induction. | 0 | 5 |
| Time matched naïve controls day 3 post AA induction. | 0 | 5 |
| Day 7 post-AA induction. | 0 | 5 |
| Time matched naïve controls day 7 post AA induction. | 0 | 5 |
| Clinical score of 1. | 1 | 7 |
| Time matched naïve controls. | 0 | 7 |
| Clinical score of 2. | 2 | 7 |
| Time matched naïve controls. | 0 | 7 |
| Clinical score of 3. | 3 | 9 |
| Time matched naïve controls. | 0 | 9 |
| Clinical score of 4. | 4 | 2 |
| Time matched naïve controls. | 0 | 2 |

Table 1. Hypoxyprobe dosing time points

Hypoxyprobe was injected intraperitoneally 6 hours before the rats were killed as determined in part 2.1.8. Their hind feet were then removed and fixed for tissue processing and subsequent histological investigations as described in parts 2.2 and Hypoxyprobe-1 adducts were detected using immunohistological method described in 2.2.9.1. The results were analysed using methods described in 2.2.10 to 2.3.

4.3 Results

The rats with adjuvant arthritis exhibited an initial weight loss during the first four days post-adjuvant induction then started to gain weight and followed a similar pattern of weight change to the naïve control rats (Figure 4). The adjuvant arthritis rats then

started to lose weight again from day 11 post-adjuvant induction until the end of the experiment on day 15. Weight loss coincided with the first clinical signs of inflammation present on day 11. Clinical score of hind feet increased to a maximum of 4, on day 15, the end of the experiment (Figure 5).

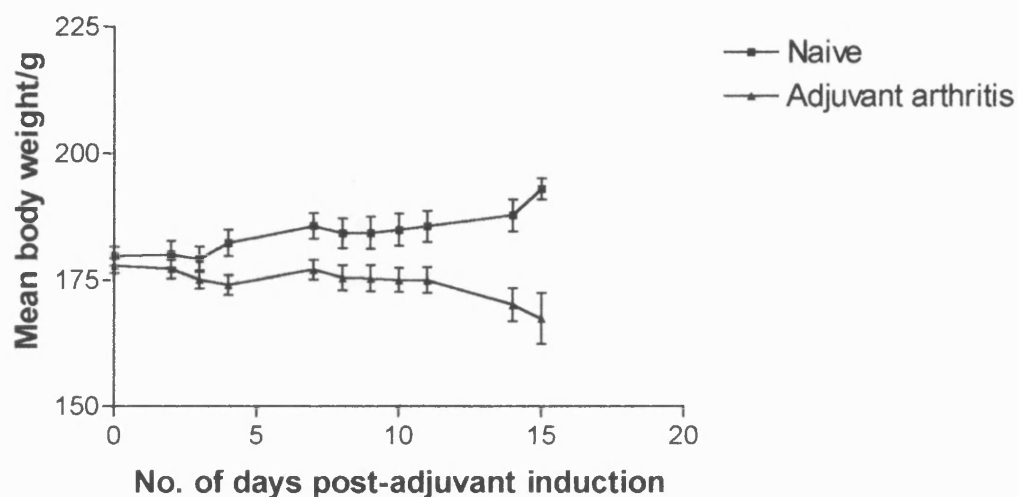


Figure 4. Changes in body weight during the development of adjuvant arthritis.

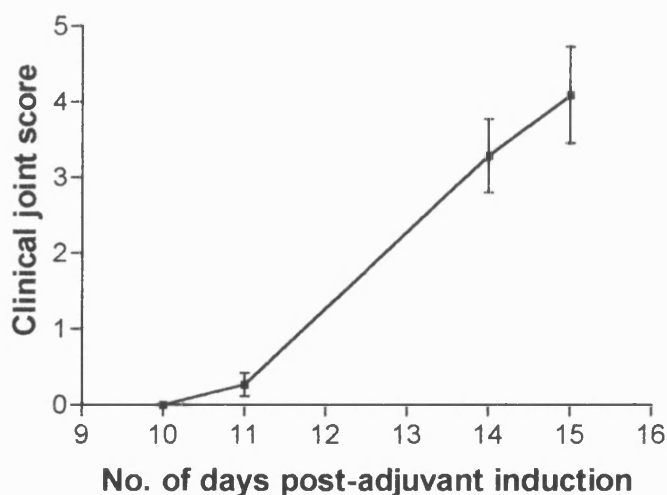


Figure 5. Development of clinical symptoms over time of adjuvant arthritis rats.

4.3.1 Results of Histological Assessment

The histological scores of the amount of synovial hyperplasia, cellular infiltration and bone erosions in the hind feet were made based on a comparison with naïve rats whose histological scores were zero (Figure 6.6). It can be seen that the inflammatory response is present on day 7 post-adjuvant induction despite the lack of clinical signs and increases to a maximum mean score of 3.5 over the subsequent 8 days. Except for onset, the histological score generally reflects the clinical score.

Plate 4.1 is a hematoxylin and eosin (H&E) stained wax section from a day 3 post-adjuvant hind foot. Histologically it is the same as its time matched naïve control (plate 4.2.) Neither show any synovitis, cellular infiltration or bone erosion. Plate 4.3 demonstrates the mild bone erosion and the hypertrophy and hyperplasia of the synovium lining layer seen in section of some day 7 adjuvant feet. Plates 4.4, 4.5, 4.6 and 4.7 are H&E wax sections from adjuvant rats with clinical scores of 1, 2, 3 and 4 respectively. These sections show an increasing level of bone erosion and more active synovitis with heavy cellular infiltration.

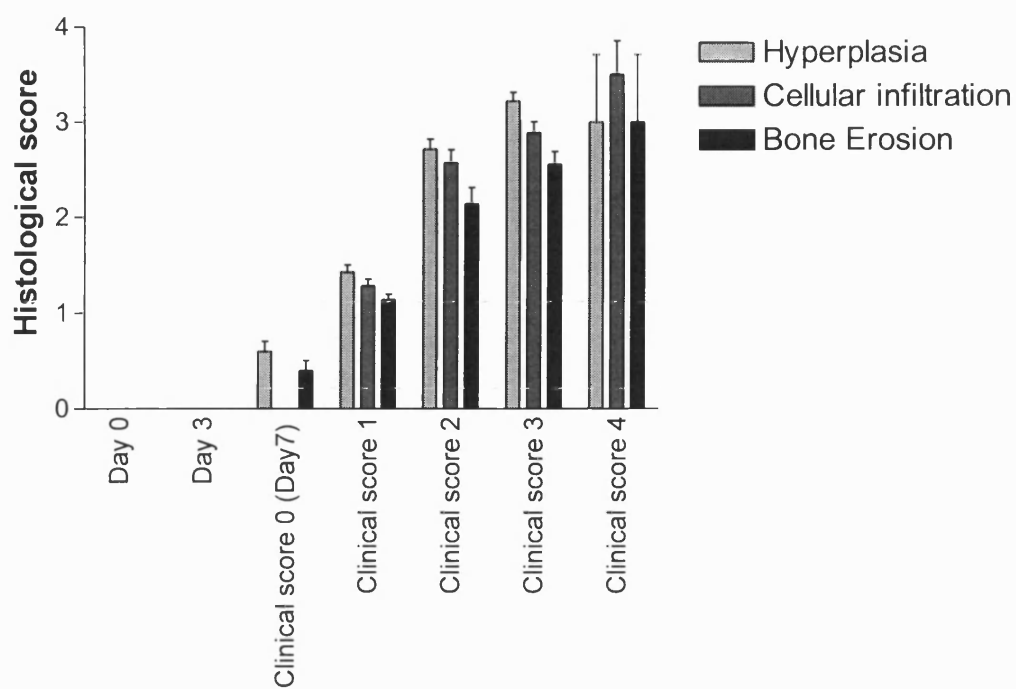


Figure 6. Change in the histological scores with development of adjuvant arthritis.

Plate 4.1 Day 3 adjuvant hind foot joint (x10, wax section, haematoxylin and eosin) a zero histological score due to absence of any cellular infiltrate and smooth appearance of the articular surface [A] and bone [B]. Also note the thin synovium [S].

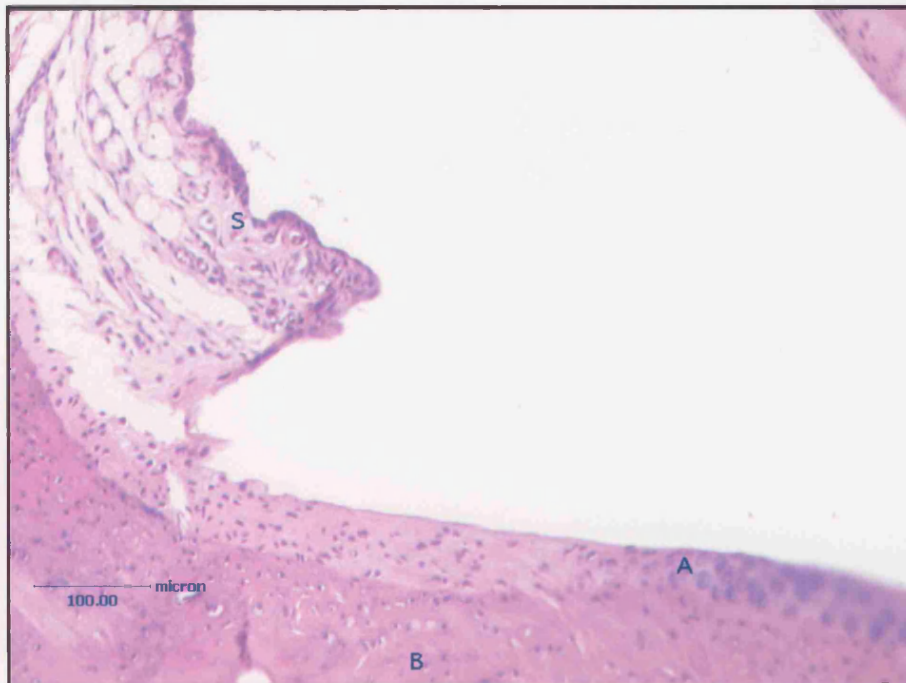


Plate 4.2 Day 3 naive hind foot joint (x10, wax section, haematoxylin and eosin) a zero histological score due to absence of any cellular infiltrate and smooth appearance of the articular surface [A] and bone [B]. Also note the thin synovium [S].

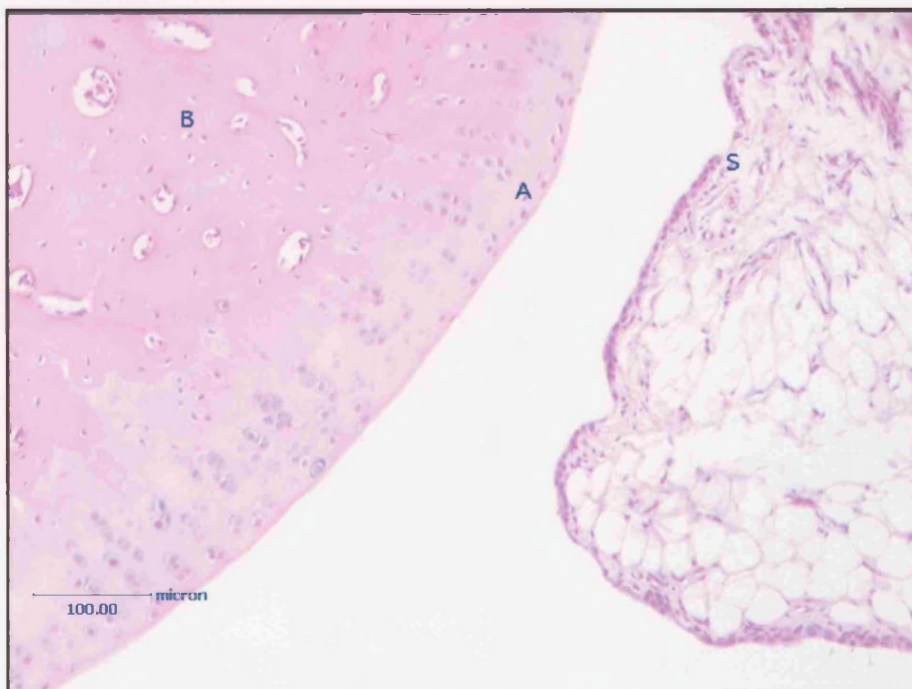


Plate 4.3 Day 7 adjuvant hind foot joint, clinical score 0. (x10, wax section, haematoxylin and eosin.) This appearance reflects a low histological score of 0-1 with some bone erosion [B] and hyperplasia and of the synovium [S].

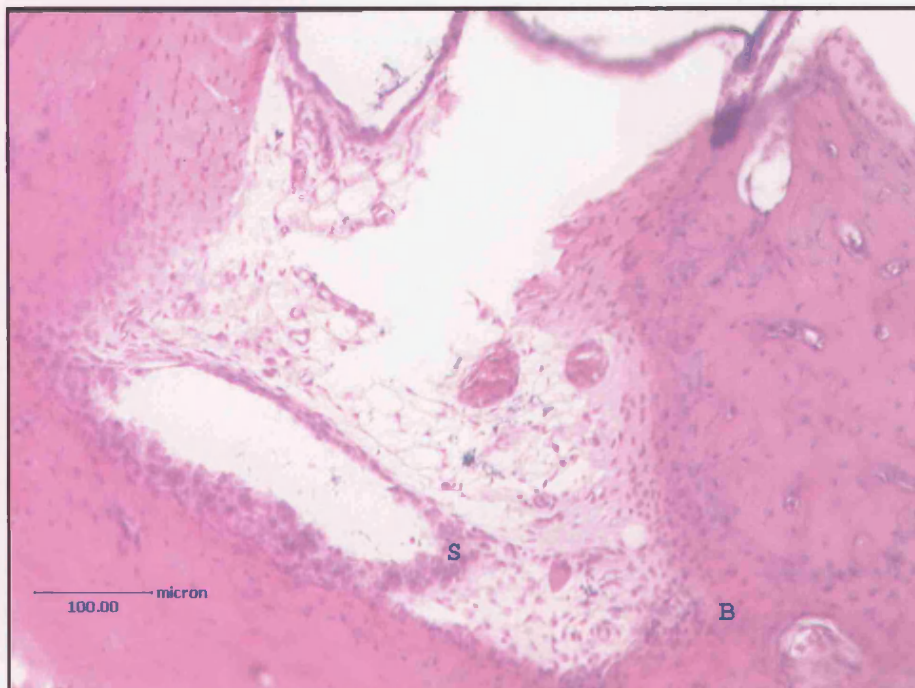


Plate 4.4 Adjuvant hind foot joint with a clinical score of 1 (x10, wax section, haematoxylin and eosin) and a low histological score 1-2 due to a mild increase in cellular infiltrate [C] and slight hyperplasia of the synovium [S].

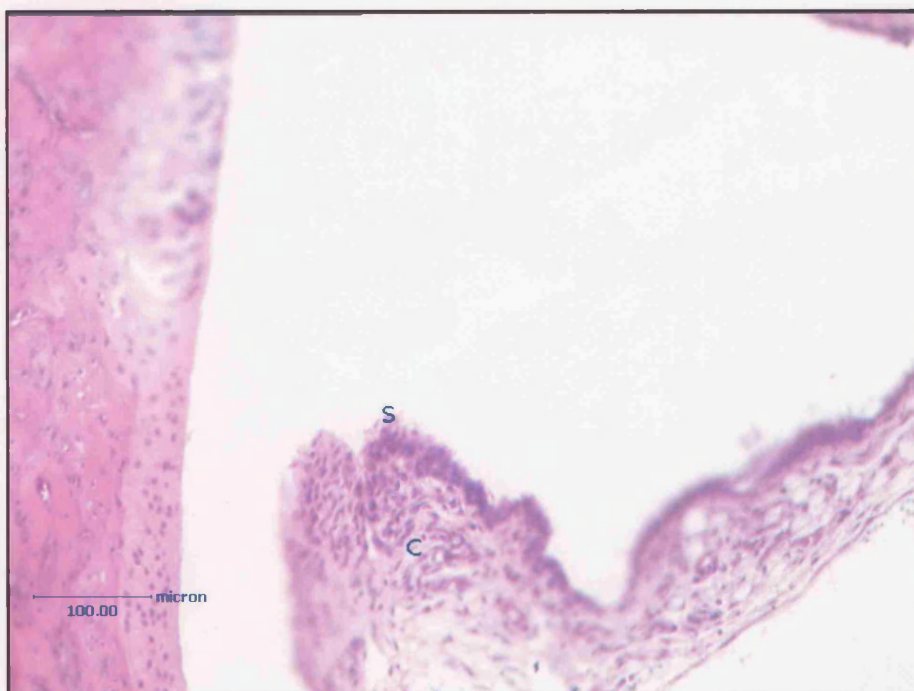


Plate 4.5 Adjuvant hind foot joint with a clinical score of 2 and a histological score of 2-3 (x10, wax section, haematoxylin and eosin). Note the increased cellular infiltrate [C].

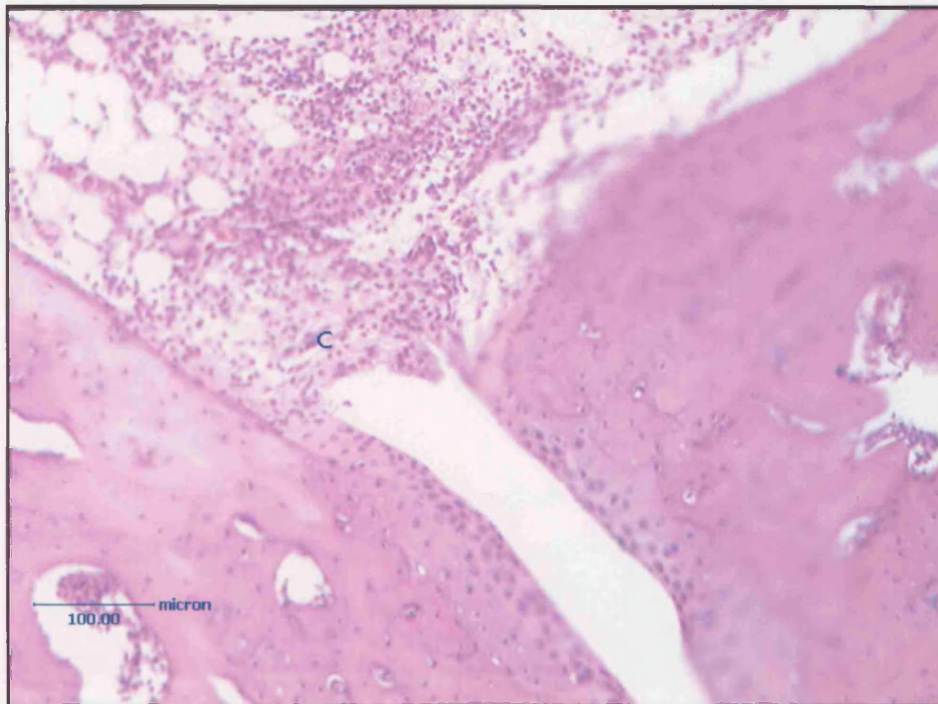


Plate 4.6 Adjuvant hind foot joint with a clinical score of 3 and a histological score of 3 (x10, wax section, haematoxylin and eosin). Note the bone erosion [B], heavy cellular infiltrate [C] and extensive hyperplasia of the synovium [S].

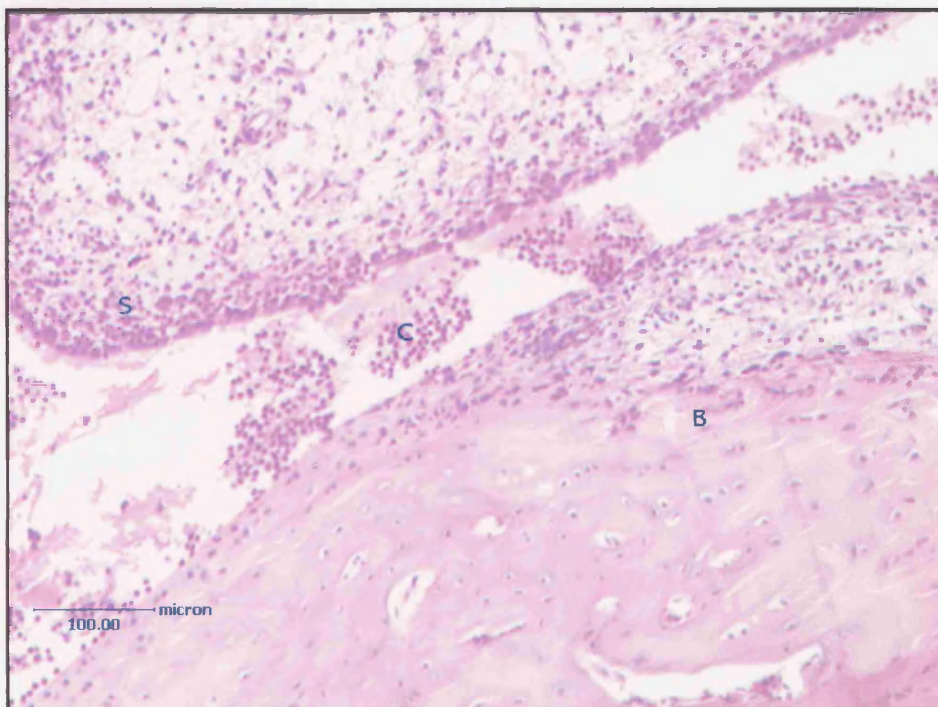


Plate 4.7 Adjuvant hind foot joint with a clinical score of 4 and a histological score of 4 (x10, wax section, haematoxylin and eosin). Note the extensive erosion of the bone [B] and heavy cellular infiltrate [C].



4.3.2 Results of Immunohistochemical assessment

There were only two samples with a clinical score of 4 so these were not used in the immunohistochemical assessment. The percentage of hypoxic-labelled area in the hind feet joints of rats with adjuvant arthritis was analysed and compared to the time matched naïve control rats (details of method in 2.4.7.)

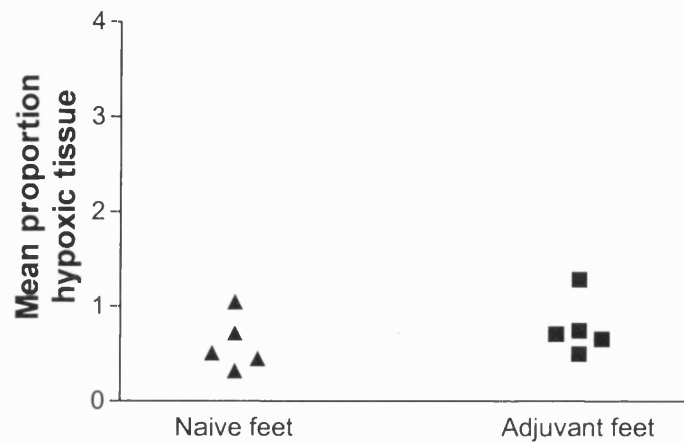


Figure 7. Day 3 post adjuvant arthritis induction. Hypoxic area of adjuvant rats (n=5) compared to naïve controls (n=5). $P>0.05$ (Mann Whitney test.)

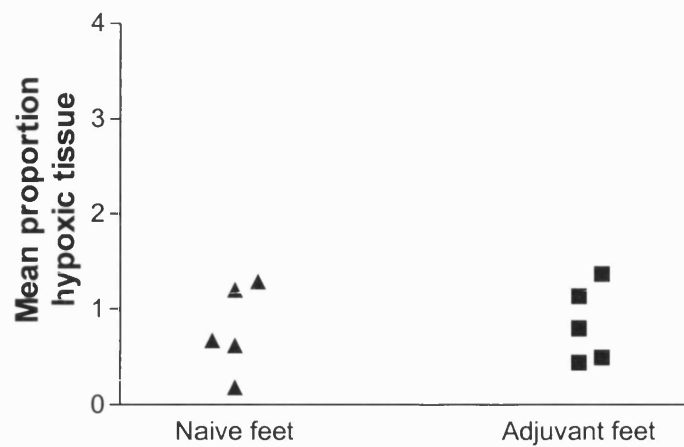


Figure 8. Day 7 post adjuvant arthritis induction. Hypoxic area of adjuvant rats (n=5) compared to naïve controls (n=5). $P>0.05$ (Mann Whitney test.).

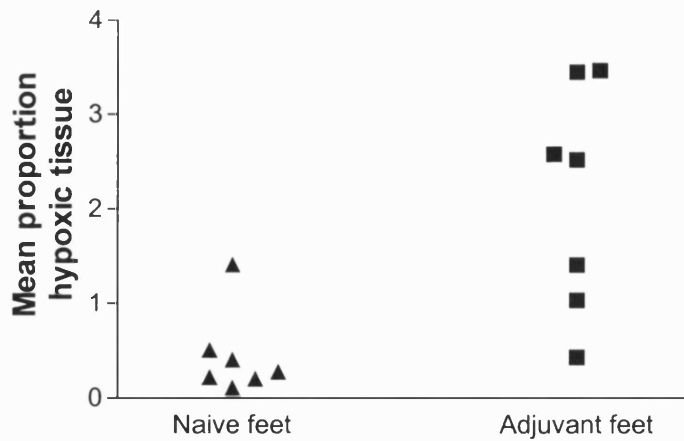


Figure 9. Hypoxic area of adjuvant rats with clinical score 1 (n=7) compared to time matched naïve controls (n=7). $P<0.001$ (Mann Whitney test.)

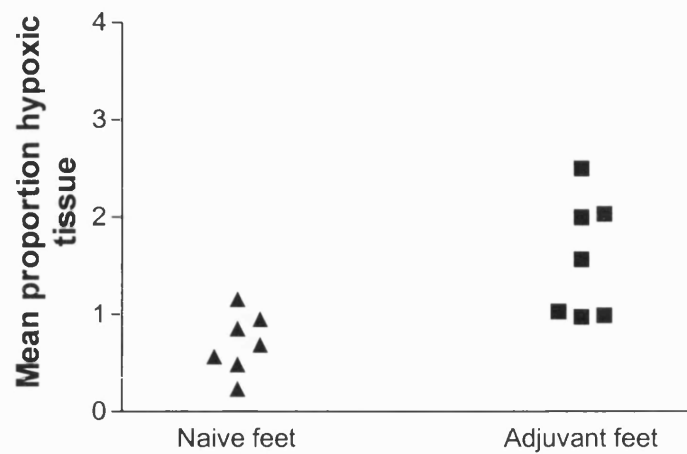


Figure 10. Hypoxic area of adjuvant rats with clinical score 2 (n=7) compared to time matched naïve controls (n=7). $P < 0.001$ (Mann Whitney test.)

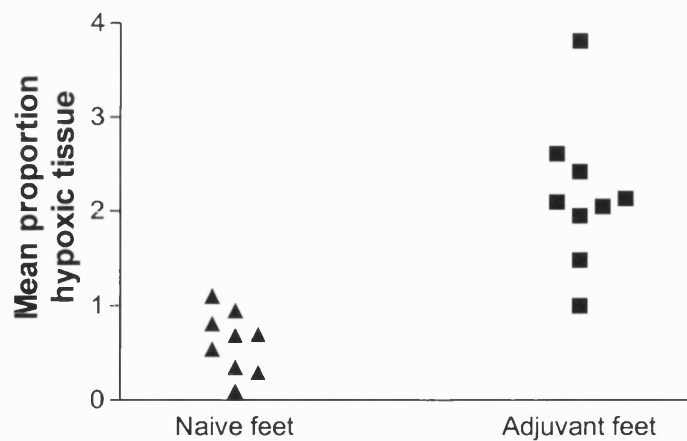


Figure 11. Hypoxic areas of adjuvant rats with clinical score 3 (n=9) compared to time matched naïve controls (n=9). $P < 0.001$ (Mann Whitney test.)

Day 0 sections (plates 4.8) from adjuvant feet were found to be histologically identical to their time-point matched naïve controls (plate 4.9) and so were not quantitatively analysed.

Day 3 and 7 adjuvant feet sections were also comparable to their naïve counterparts (plates 4.10, 4.11, 4.12 and 4.13). The adjuvant feet showed minimal staining for hypoxypromide in the fatty area of the synovium and in a few areas of the lining cells of the synovium. This similarity between the groups was reflected in the data in Figure 7 and Figure 8, which showed no significant difference in levels of staining for hypoxypromide between the adjuvant rats and their time-point matched naïve controls, $P>0.05$ (Mann Whitney test.)

Figure 9, shows that when the first clinical signs of disease appear clinical score 1 (C1) after 11 days post adjuvant induction there is a concordant increase in the area staining for hypoxypromide in the adjuvant rats. Statistical analysis of the data shows this to be highly significant, $P<0.001$ (Mann Whitney test.) The staining for hypoxypromide was found mainly in the proliferating synovium of C1 hind feet and clusters of cells within the synovium were found to stain specifically. These cell clusters were often associated with blood vessels as demonstrated in plates 4.14 and 4.15 and were not found in any sections of naïve controls (plate 4.16). The data (Figure 10) for clinical score 2 (C2) adjuvant hind feet also show an increase in staining for hypoxypromide compared to their time-point matched naïve controls that is highly significant, $P<0.001$ (Mann Whitney test.) The very different staining levels are seen in plates 4.17 and 4.18 respectively.

Staining for hypoxypromide in chondrocytes of adjuvant rats with clinical symptoms was greatly increased and this can be seen in plates 4.19 and 4.20. By comparison only pale staining for hypoxypromide was present in chondrocytes from naïve control hind feet taken at all time points. An example of this is shown in plate 4.21. Finally, adjuvant rats with a clinical score of 3 (C3) were found to have the highest levels of staining for

hypoxypromide (Figure 11). Again the difference between the C3 rats and their time-point matched naïve controls was highly significant, $P < 0.001$ (Mann Whitney test.) Staining was found in most of the tissue structure involved in synovitis and bone erosion. There was staining in the bone marrow (plate 4.22), chondrocytes (plate 4.20) and synovium (plate 4.23). This intensity of staining was not found in naïve sections an example is seen in plate 4.24.

Plate 4.8 Day 0 adjuvant hind foot joint (x10, wax section, stained with mouse anti-hypoxyprobe). Strong specific red staining is absent from the articular cartilage [A] and the synovium [S].

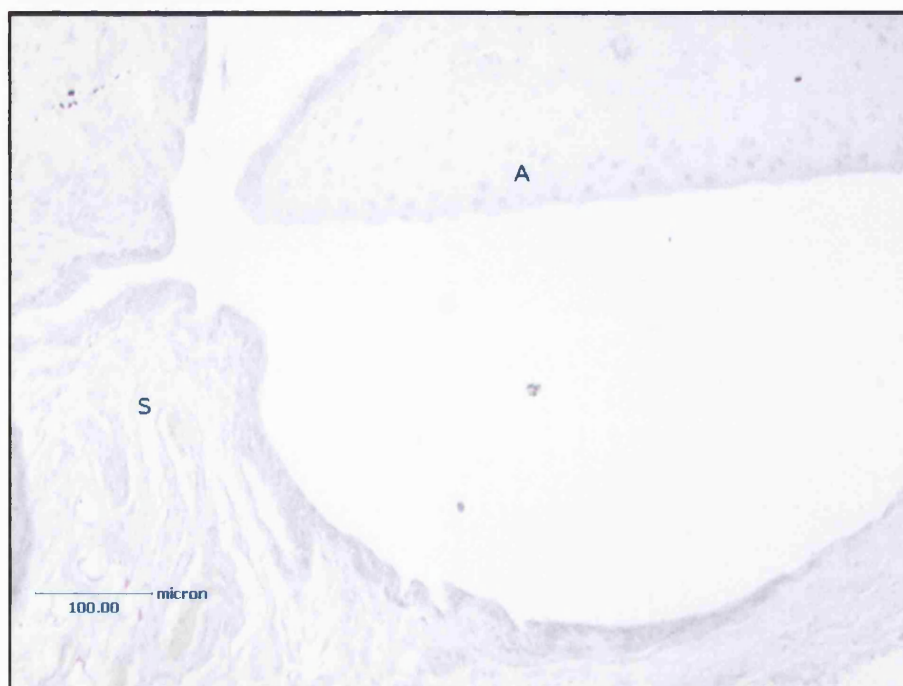


Plate 4.9 Day 0 naive hind foot joint (x10, wax section, stained with mouse anti-hypoxyprobe). No strong specific red stain in articular cartilage [A] the synovium [S].

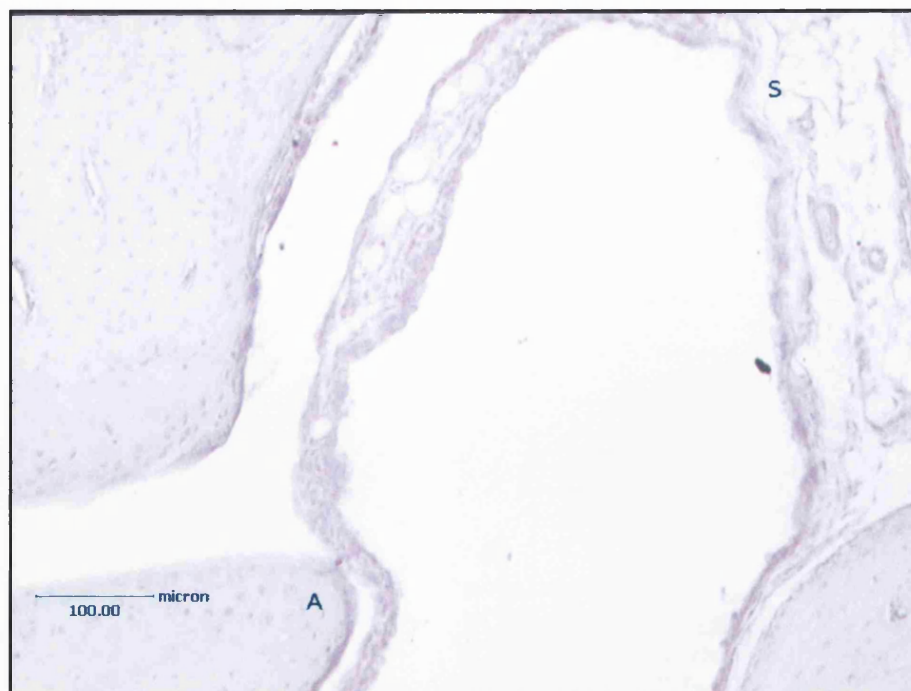


Plate 4.10 Day 3 adjuvant hind foot joint (x10, wax section, stained with mouse anti-hypoxypore). Articular cartilage [A]. There is pale staining in the fatty area of the synovium [S].

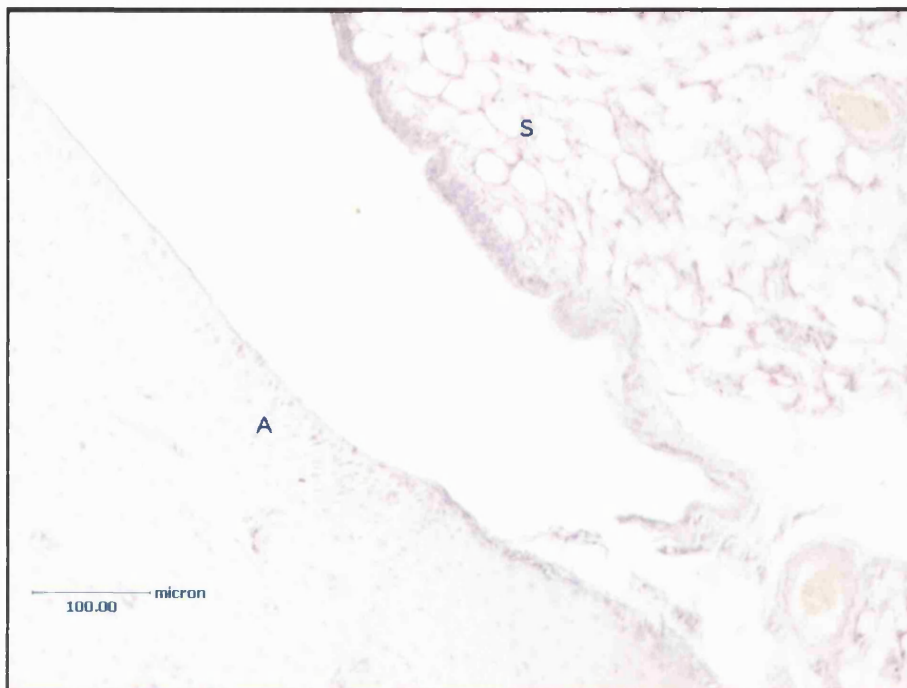


Plate 4.11 Day 3 naive hind foot joint (x10, wax section, stained with mouse anti-hypoxypore). Bone [B]. Note the stain in the synovium [S] and pale staining in the chondrocytes [arrows.]

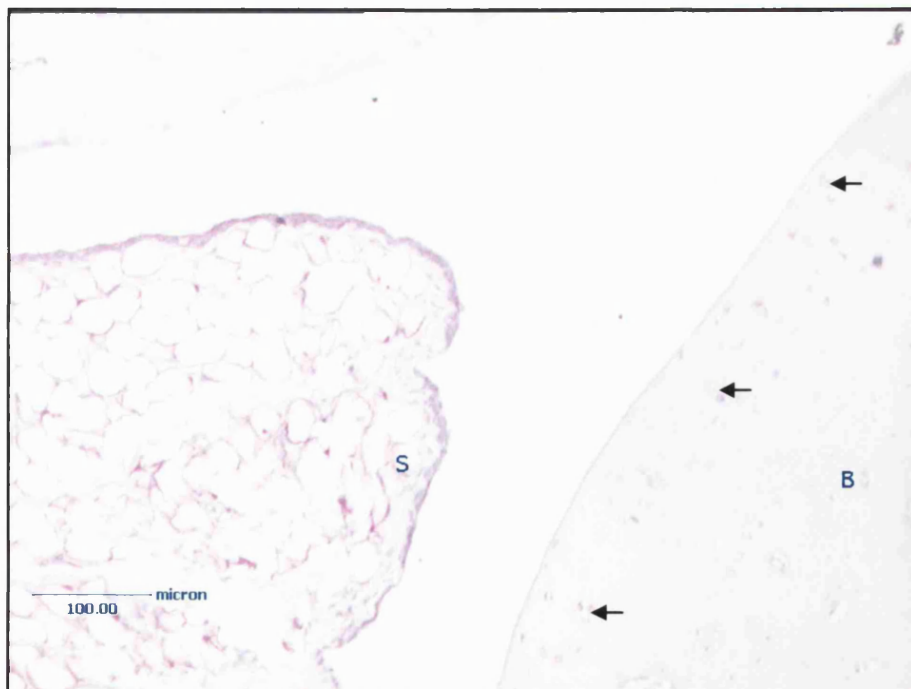


Plate 4.12 Day 7 adjuvant hind foot joint (x10, wax section, stained with mouse anti-hypoxyprobe). Bone [B]. The lining cells of the synovium [S] are weakly stained.

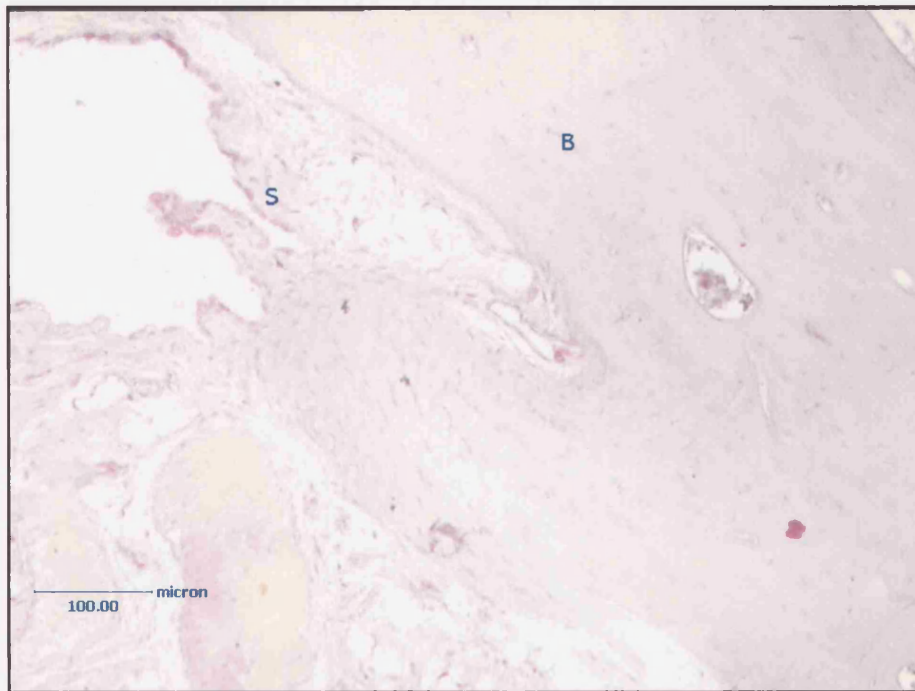


Plate 4.13 Day 7 naive hind foot joint (x10, wax section, stained with mouse anti-hypoxyprobe). Note the absence of red stain in the bone [B] and synovium [S].

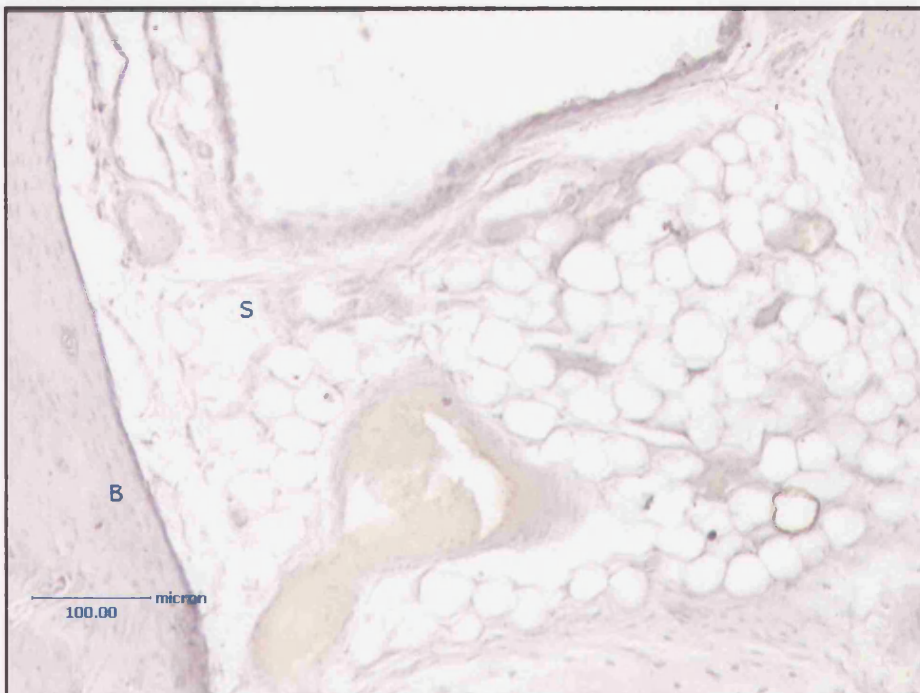


Plate 4.14 Adjuvant hind foot joint with a clinical score of 1 (x10, wax section, stained with mouse anti-hypoxyprobe). Bone [B]. There is strong red specific staining of cells [arrows] in the synovium [S] near blood vessels [BV].

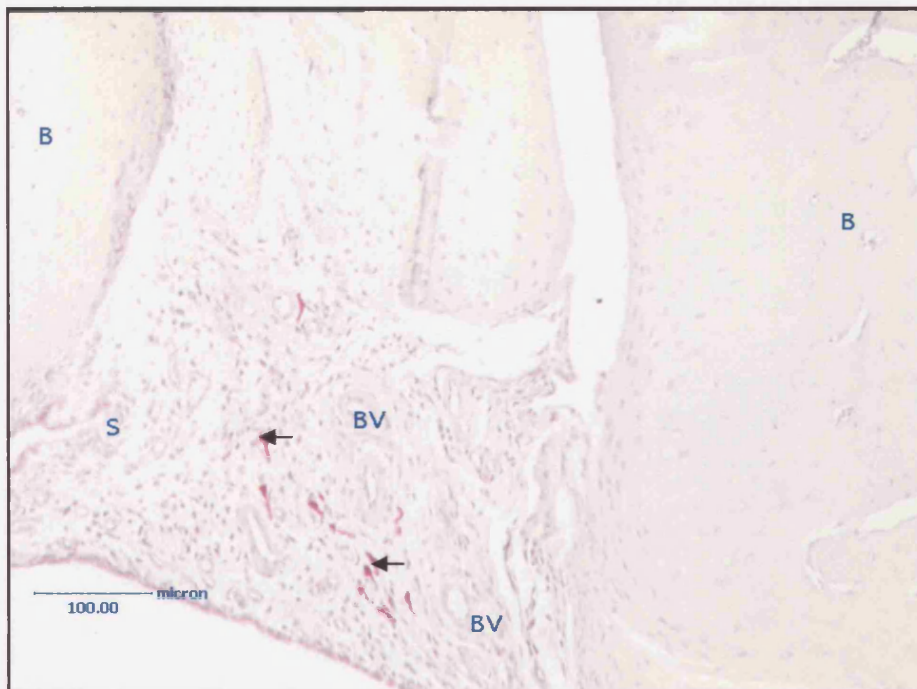


Plate 4.15 Adjuvant hind foot joint with clinical score of 1 (x20, wax section, stained with mouse anti-hypoxyprobe). Erosion of bone [B] is visible at this higher magnification and also strong specific staining of cells [arrows] in the synovium [S] near blood vessels [BV].

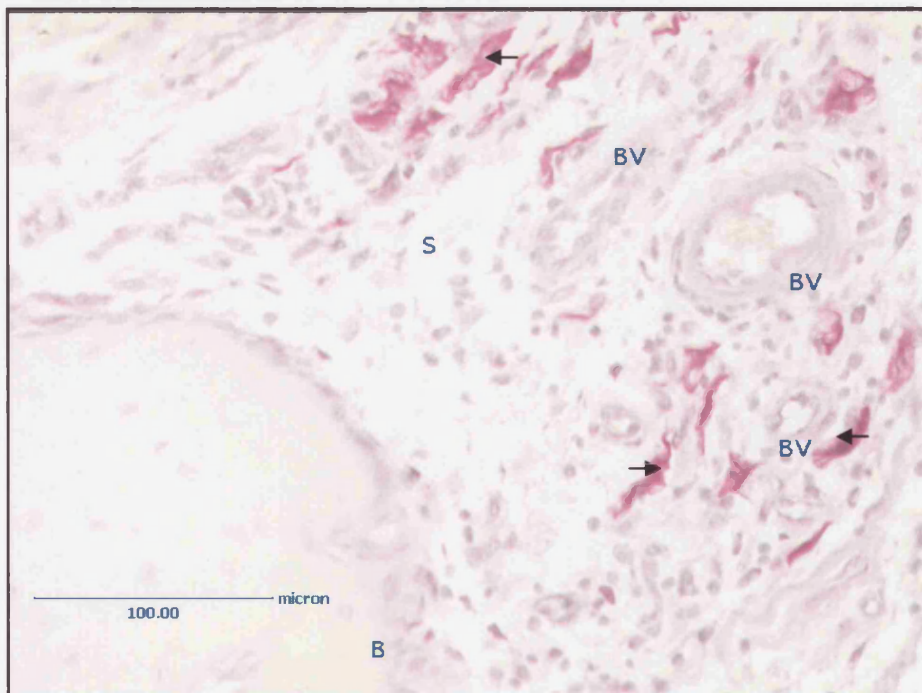


Plate 4.16 Naive hind foot joint time matched (C1) control (x20, wax section, stained with mouse anti-hypoxyprobe). Articular cartilage [A], blood vessels [BV]. There is no cellular infiltrate or stained cells in the synovium [S].

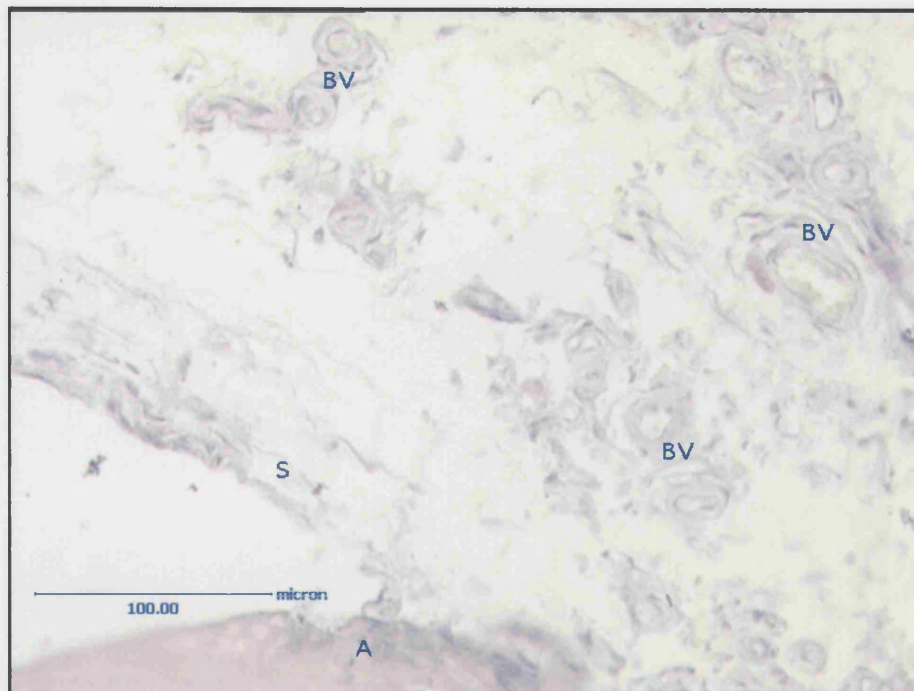


Plate 4.17 Adjuvant hind foot joint with clinical score of 2 (x5, wax section, stained with mouse anti-hypoxyprobe). Bone [B]. Positive red staining is present in the chondrocytes in articular cartilage [A}.

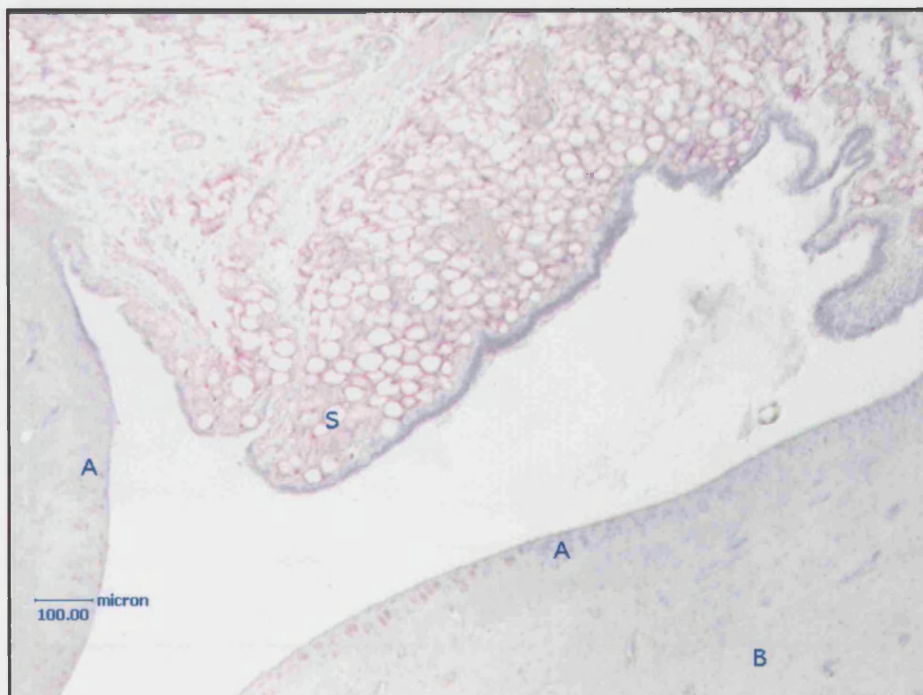


Plate 4.18 Naive hind foot joint time matched (C2) control (x5, wax section, stained with mouse anti-hypoxyprobe). Articular cartilage [A], blood vessels [BV]. Note the absence of cellular infiltrate and stained cells in the synovium [S].

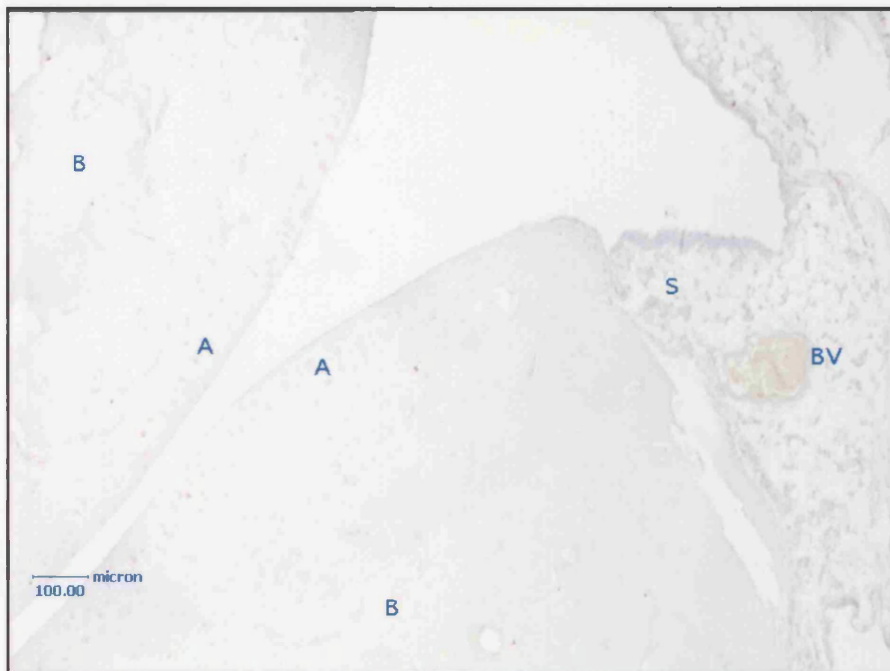


Plate 4.19 Adjuvant hind foot joint with clinical score of 2 (x40, wax section, stained with mouse anti-hypoxyprobe). Strong specific staining of chondrocytes [arrows] in the articular cartilage [A].

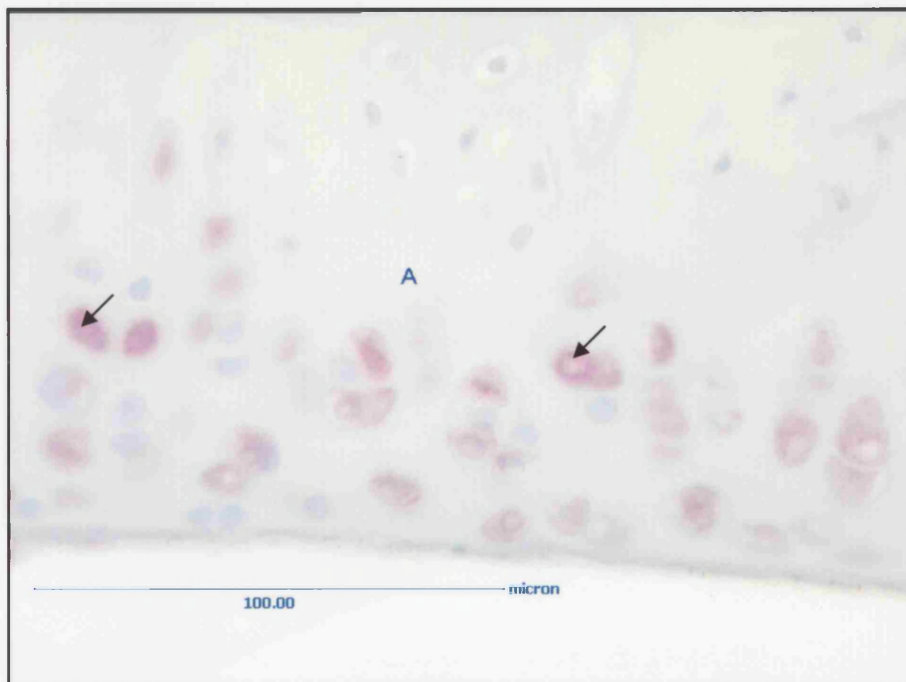


Plate 4.20 Adjuvant hind foot joint with clinical score of 3 (x20, wax section, stained with mouse anti-hypoxypore). Strong specific staining of chondrocytes [arrows] in the articular cartilage [A].

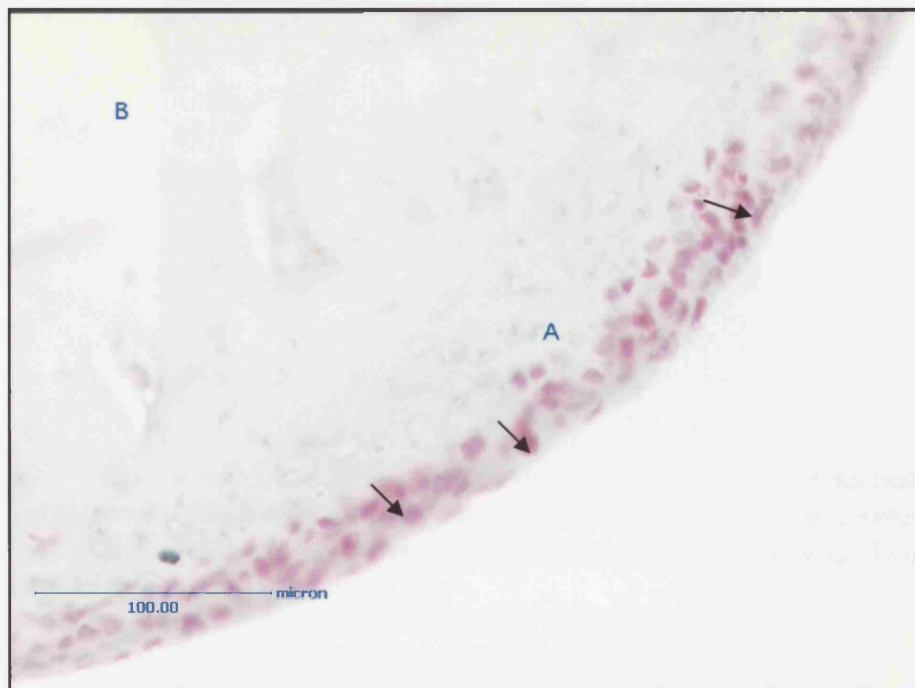


Plate 4.21 Naïve hind foot joint time matched (C3) control (x20, wax section, stained with mouse anti-hypoxypore). Bone end is shown [B]. Weak staining of chondrocytes [arrows] in the articular cartilage [A].

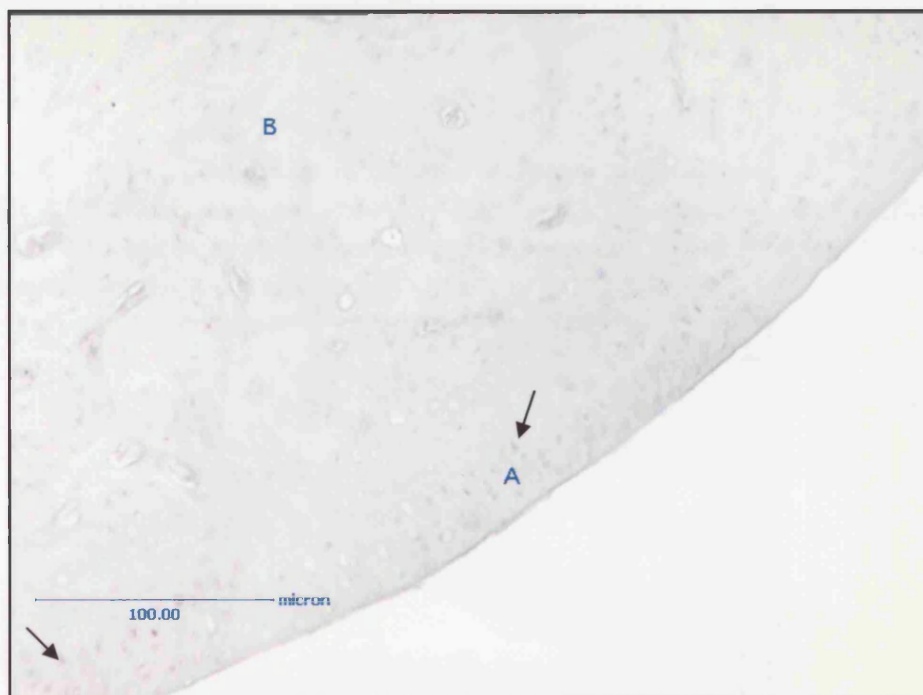


Plate 4.22 Adjuvant hind foot joint with clinical score of 3 (x10, wax section, stained with mouse anti-hypoxyprobe). There is erosion of bone [B] by invasive synovium [S] and specific staining of chondrocytes in the articular cartilage [A].

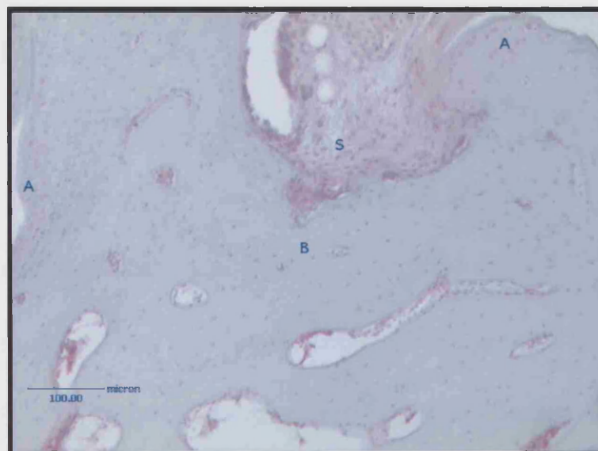


Plate 4.23 Adjuvant hind foot joint with clinical score of 3 (x40, wax section, stained with mouse anti-hypoxyprobe). Erosion of bone [B] by invasive synovium [S] is well shown at this magnification. Note the strong specific staining of cells in the synovium [arrows].

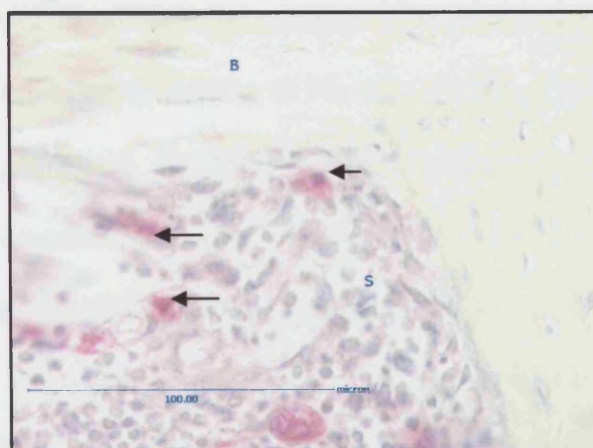
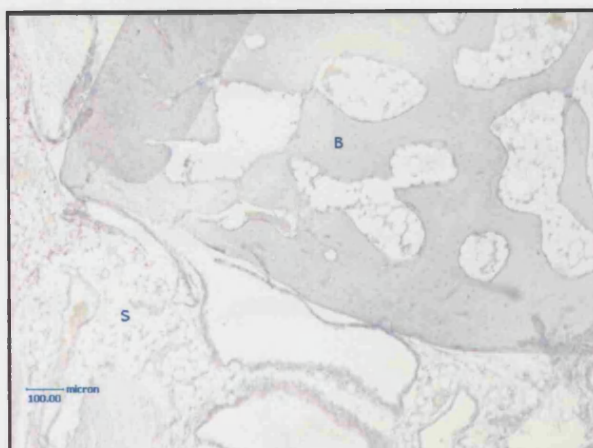


Plate 4.24 Naive hind foot joint time matched (C3) control (x5, wax section, stained with mouse anti-hypoxyprobe). Bone erosion [B] is absent and bone and synovium [S] lack staining.



4.4 Discussion.

As discussed in the introduction, the rat adjuvant arthritis model is useful in investigating the pathology and novel treatment of rheumatoid arthritis (RA). Many features of the development and pathology of adjuvant disease in rats are similar to those seen in humans with RA. The clinical signs of joint swelling and destruction discussed in this thesis are typical of published descriptions of adjuvant disease (Stoerk et al., 1961; Pearson et al., 1961) and are comparable to the clinical-pathological changes seen in RA joints. Examination of the joint synovium from both RA patients and rats with adjuvant arthritis shows that both undergo hyperplasia and substantial cellular infiltration with the eventual destruction of bone and cartilage (Cush & Lipsky, 1991; Fitzgerald & Bresnihan, 1995; Pelegri et al., 1995; Zvaifler & Firestein, 1994).

Also previously reviewed in the introduction were studies that demonstrated hypoxia in rheumatoid but not non-rheumatoid joints (Falchuk et al, 1970; Lund-Oleson, 1970; Treuhaft and McCarty, 1971; Blake et al, 1997). This work has also been supported by previous departmental studies in the rat model of adjuvant arthritis that have demonstrated a significant decrease in pO_2 between day seven and fourteen post adjuvant arthritis induction (Bruce, 1992). None of these studies identify or describe specific hypoxic cell populations within the joint.

Hypoxyprobe is a commercially available preparation of pimonidazole which following dosing, is distributed to all tissues in the body but binds only to cells that have low oxygen concentrations equivalent to a pO_2 of 10 mm Hg (or less) at 37°C. Hypoxyprobe-1MAb1 is a monoclonal antibody that detects protein adducts of

Hypoxyprobe produced in hypoxic cells. The reductive metabolism of this hypoxia marker is regulated by oxygen tension independent of pyridine nucleotide redox state (Arteel et al, 1998). Pimonidazole was first used to measure low oxygen in animal and human tumours (Raleigh et al., 1987; Azuma et al., 1997; Kennedy et al, 1997). My study represents the first use of a definitive exogenous marker, Hypoxprobe-1, to detect hypoxic cells in the inflamed joint *in vivo*. My results show for the first time that inflamed joints in the rat adjuvant model possess significantly more hypoxic tissue, as demonstrated by positive hypoxyprobe-1 adduct immunoreactivity, than those from naïve controls. I have shown that as the rats develop clinical signs of adjuvant arthritis the percentage of tissue area staining for hypoxyprobe measured quantitatively increases significantly compared to naïve controls. Qualitative assessment of the distribution of the hypoxyprobe shows that as adjuvant disease progresses tissue hypoxia involves all tissue structures including the synovium, bone marrow and articular cartilage chondrocytes. Of particular interest are the clusters of yet to be identified cells within the proliferating synovium that stain intensely with hypoxyprobe. These cells are unique to the adjuvant synovium and were not found in the synovium of naïve rats. These cells were found near blood vessels, therefore it may be asked why are these cells hypoxic. The reason could be explained by two different mechanisms. The area may be subject to hypoxic episodes as a result of hypoxic reperfusion events previously described in the introduction (section 1.8). Therefore this staining represents a “snapshot” of a period of hypoxia. The alternative suggestion is that these cells are migratory and have migrated from an area of hypoxia the the area near the blood vessels which is not hypoxic. I suggest these cells might have the phenotype of the “transformed” aggressive, invasive fibroblast-type cells previously described in section 1.2.5

of the introduction. To date no single specific marker for this phenotype has been identified. Instead a combination of characteristics has been observed which lead to the cells aggressive, invasive behavior. These 'transformed cells' respond to stimulation by cytokines and growth factors produced in the local inflammatory milieu but also their cellular changes are maintained in the absence of external stimulation (Pap et al 2000). Alternatively the clusters of cells labeling with hypoxypromote might be monocytes recruited from the inflammatory milieu.

Shapiro et al (1997) used EF5 a pentafluorinated derivative of 2-nitroimidazole (etanidazole) that is metabolically reduced by nitroreductases to label hypoxic chondrocytes. In their study chondrocytes generally had low levels of EF5 binding; however, exposing chondrocytes to hypoxic conditions significantly increased EF5 binding. My work supports these findings as I have shown staining for hypoxypromote in chondrocytes of the naïve controls was pale compared to the intense staining seen in chondrocytes of the adjuvant group.

Chondrocytes can adapt to conditions of low oxygen tensions by their capacity to switch to anaerobic metabolism (Rajpurohit et al., 1996). It is possible that the hypoxia in chondrocytes may be a result of pannus development since the increase in metabolic demand of the cells in the developing pannus and poor vascular supply may compromise the metabolic requirements of the chondrocytes. The levels of hypoxia indicated by the hypoxypromote staining in adjuvant cartilage chondrocytes could bring about deleterious changes in these cells. In 1985 Mitrovic suggested that chondrocytes contribute to the formation of the pannus. Chondrocytes activated by inflammatory cytokines dedifferentiate, enlarge their lacunae, coalesce and finally break onto the cartilage surface

to form a fibrous pannus. The fibrous pannus tissue is subsequently invaded by inflammatory cells to form the inflamed synovium. (Mitrovic 1985).

4.5 Conclusions

These results show, for the first time, as the clinical symptoms of adjuvant arthritis develop so the inflamed joint becomes hypoxic and that this hypoxia is significantly higher than naïve controls. Further work is needed to identify the cell types that are labelling with hypoxyprobe-1 adduct immunoreactivity.

CHAPTER 5: Hypoxia, Cell Characterisation, Transcription factors and Cytokines.

5.1 Introduction

The previous chapter demonstrated that as the clinical symptoms of adjuvant arthritis develop so the cells within the inflamed joint become hypoxic and that this tissue hypoxia is significantly higher than in naïve controls. The principle aim of this chapter is to identify the cell types that are found in the hypoxic areas (as demonstrated by hypoxyprobe adduct detection). The overall hypothesis of this thesis is that hypoxic cells in the inflamed joint produce proinflammatory cytokines and provide a potential target for bio-reductive therapy and other novel therapies. Therefore, the second part of this chapter describes studies aiming to show whether hypoxic cells produce proinflammatory factors. To do this several key markers were examined: iNOS, VCAM-1, VEGF, HIF-1 α and ets-1. As previously described in the introduction (section 1.9.3) inducible nitric oxide synthase (iNOS) is considered a proinflammatory enzyme because of its ability to form damaging free radicals and it is highly expressed in the rheumatoid synovium (Sakurai et al., 1995, Gabowski et al., 1997). Vascular cell adhesion molecule-1 VCAM-1 is a member of a family of cell adhesion molecules that are responsible for the recruitment of leukocytes from the circulatory system to a site of inflammation. This process occurs via a multistep pathway which involves binding of leukocytes to the vascular endothelium, a rolling mechanism, and finally a firm adhesion followed by passage of the leukocyte between endothelial cells and into the tissue (diapedesis). VCAM-1 is an endothelial ligand responsible for the initial binding of leukocytes to the endothelium. Increased VCAM-1 expression has been demonstrated in the synovium from RA patients (Morales-Ducret et

al., 1992). Also, soluble VCAM-1 has been implicated in exacerbation of rheumatoid arthritis through the induction of angiogenesis (Koch et al., 1995). The cytokine, vascular endothelial growth factor (VEGF), is considered a potent inducer of angiogenesis (Brenchley, 2000). It is constitutively secreted by rheumatoid synovial fibroblasts and can be increased *in vitro* when the synoviocytes are exposed to hypoxia (Jackson et al., 1997). As mentioned in section 1.9.4, HIF-1 α is the oxygen regulated subunit of the transcription factor HIF-1 (Jiang et al., 1996; Semenza, 2000) and its expression by macrophages has been demonstrated in the rheumatoid synovium (Hollander et al., 2001). Therefore the distribution of HIF-1 α in the inflamed joint would be expected to colocalize with the distribution of hypoxic cells detected by hypoxyprobe. There are a number of genes that are regulated by HIF-1 of particular relevance to rheumatoid arthritis are: iNOS, VEGF and PDGF (Ratcliffe et al., 1998).

Finally, the transcription factor, Ets-1, belongs to the Ets family of transcription factors defined by a conserved DNA-binding Ets domain that forms a winged helix-turn-helix structural motif. Ets-1 has been identified in endothelial cells during angiogenesis both in the embryo (Vandebunder et al., 1989) and in tumours (Wernert et al., 1992). C-Ets-1 expression in cancer cells has been correlated with tumour invasion (Wernert et al., 1994) and it has been shown *in vitro* that hypoxia induces Ets-1 via HIF-1 in a human bladder cancer cell line (Oikawa et al., 2001).

At the time this part of my thesis study was carried out, publications on ets-1 in rheumatoid arthritis were sparse and little was known except that over-expression of Ets-1 has been observed in cultures of TNF α -stimulated synovial fibroblasts from RA synovial tissue (Redlich et al., 2001). Therefore, the objective of this study was to determine the

relationship between hypoxia and expression of the transcription factors, ets-1 and Hypoxia Inducible Factor-1 α (HIF-1 α), and cytokines in both normal and inflamed joints of adjuvant-induced arthritis (AA) as a model that mirrors many aspects of rheumatoid arthritis.

5.2 Materials and Methods

20 Female Lewis Rats were caged in groups of five. Adjuvant arthritis was induced in 10 as described in part 2.1.5. and 10 were left as non-diseased controls. The disease progression was monitored by measuring body weight daily and clinically scoring the hind limbs according to the method of Andrews et al. (1987a,b) The method is fully described in section 3.1.6. When a maximum clinical joint score of 4 was achieved in total of two feet scores in the adjuvant group, all 20 rats were killed to comply with Home Office regulations which state that the animals must be killed when one foot reaches a score of 4. Hypoxyprobe was injected intraperitoneally 6 hours before the rats were killed as described in part 2.1.8. The rats were killed by cervical dislocation and their hind feet removed and fixed prior to tissue processing and histological investigations as described in chapter 2.2. Knee synovium was also removed and snap frozen as described in 2.2.6. 5 μ m thick alternate downward and upward facing (flip-flop) and consecutive wax sections were cut, mounted on glass slides and dried in an oven at 60°C overnight. 10 μ m thick consecutive frozen sections were cut, mounted on glass slides and dried for 1 hour before use. Hypoxyprobe-1 adducts were detected using immunohistological method described in 2.2.9.1. VCAM-1, VEGF, iNOS, fibroblast and macrophage markers were detected using the immunohistological method described in 2.2.9.2. Transcription factor ets-1 and HIF-1 α

expression was revealed using immunohistological methods described in 2.2.9.2 and 2.2.9.3 respectively. The results were analysed using methods described in 2.2.10 to 2.3. Image analysis was limited to synovial tissue lining the joint cavity and synovial tissue attached to the bone. The KS 300 Imaging System V 3.0 was used to count nuclei with positive immunoreactivity for Ets-1 to be expressed as a proportion of the total cell population counted in a framed area of 100 x 100 μ m. The proportion of nuclei showing immunoreactivity for both Ets-1 and HIF-1 α was also assessed. This data was then tabulated using software (Graph Pad) for statistical analysis.

5.3 Results

5.3.1 Results of Histological Assessment

In the rat adjuvant model used in this study, marked changes were visible in inflamed joints within 10 days of disease induction. Clinically detectable swelling was present in the distal joints which increased in severity and led to ankle joint deformation by day 13. Histological examination of adjuvant rat foot sections revealed synovial hyperplasia, increased cellular infiltration and bone destruction. The disease progression and histological changes were reflected those seen in chapter 4.

5.3.2 Results of Immunohistochemical Assessment.

5.3.2.1 Results of cell type, cytokine, hypoxia and transcription factor markers in fresh frozen synovial tissue.

None of the antibodies worked effectively enough in fresh frozen sections to enable data interpretation. A summary of observations is available below in Figure 12.

| Marker | Result |
|--------------------|---------------------------------------|
| Fibroblast (5B5) | No stain |
| Macrophage (ED2) | No stain |
| Macrophage (ED1) | High background and no specific stain |
| INOS | High background and no specific stain |
| VEGF | No stain |
| VCAM-1 | No stain |
| Hypoxypore (1MAB1) | No stain |
| HIF-1 α | High background and no specific stain |
| Ets-1 | No stain. |

Figure 12. Summary of immunohistochemical results in fresh frozen tissue.

5.3.2.2 Results of cell type, cytokine, hypoxia and transcription factor markers in wax embedded tissue

Three types of negative controls for the various antibodies were carried out, all were clear of staining:

- 1.) Plate 5.1. Sections from adjuvant arthritis hind feet using an Irrelevant primary antibody, mouse anti *Aspergillus niger* glucose oxidase.
- 2.) Plate 5.2. Sections from adjuvant arthritis hind feet using goat IgG.
- 3.) Plate 5.3. Sections from adjuvant arthritis hind feet using rabbit IgG.

Two markers failed to work in adjuvant rat wax sections these were: Fibroblast marker 5B5 and VCAM-1.

ED1 macrophage marker labels a protein found on the lysosomal membrane of myeloid cells. It labels strongly in most tissue macrophages and weakly in peripheral granulocytes. It shows homology to human CD68 macrophage marker. ED2 labels a protein on peritoneal macrophages but not monocytes or microglial cells. Plates 5.4 and 5.5 show staining for ED1 and ED2 respectively in naïve hind feet. It can be seen that staining for ED1 is sparse and mainly in the lining cells of the synovium. By comparison ED2 stains more cells but again limited mainly to the synovial lining cells. Plates 5.5 and 5.6 show staining for ED1 and ED2 respectively in adjuvant hind feet. It can be seen that more cells stained for ED1 in the adjuvant joints compared to naïve joints and in cells in the depths of the synovium. Again, more cells in the adjuvant joint stained ED2 compared naïve joint staining and it appears that there is a proliferation of ED2 labelling cells deeper in the synovium. Areas of increased staining did not colocalize specifically with areas of hypoxia (as shown by hypoxyprobe adduct detection).

5.3.2 Results

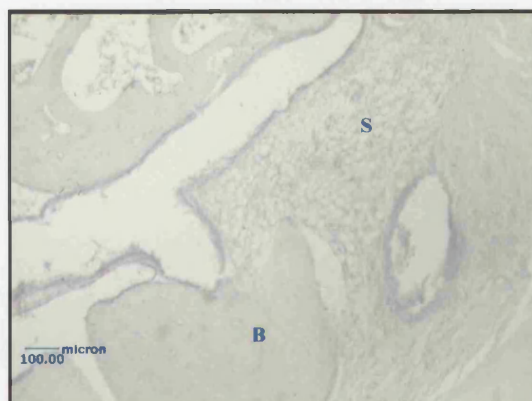


Plate 5.1 Negative control:
Adjuvant hind foot joint,
(x10,wax section, stained using
irrelevant mouse antibody). Bone
[B] and synovium [S].

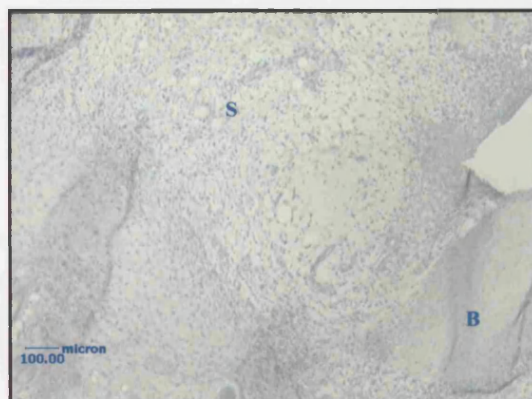


Plate 5.2 Negative control:
Adjuvant hind foot joint, (x10,wax
section, stained using goat IgG.)
Bone [B] and synovium [S].



Plate 5.3 Negative control:
Adjuvant hind foot joint, (x10,wax
section, stained using rabbit IgG.)
Bone [B] and synovium [S].

Plate 5.4 Naive hind foot (x10, wax section, stained with mouse anti-rat ED1). Articular cartilage [A]. A thin pattern of stained cells on the lining of the synovium [S].

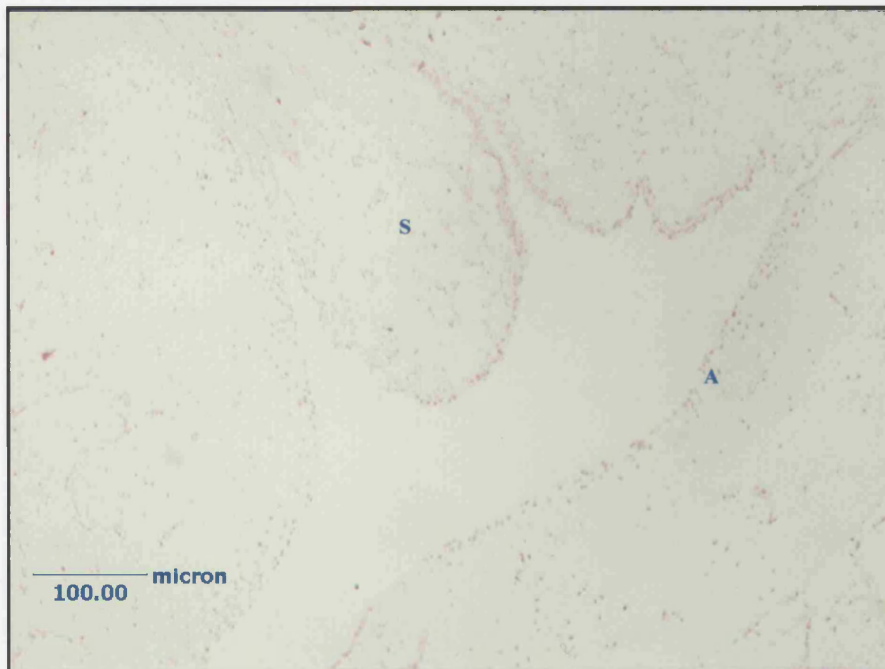


Plate 5.5 Naive hind foot (x10, wax section, stained with mouse anti-rat ED2). Articular cartilage [A]. The pattern of stained cells on the lining of the synovium [S] is thicker compared to plate 6.4.

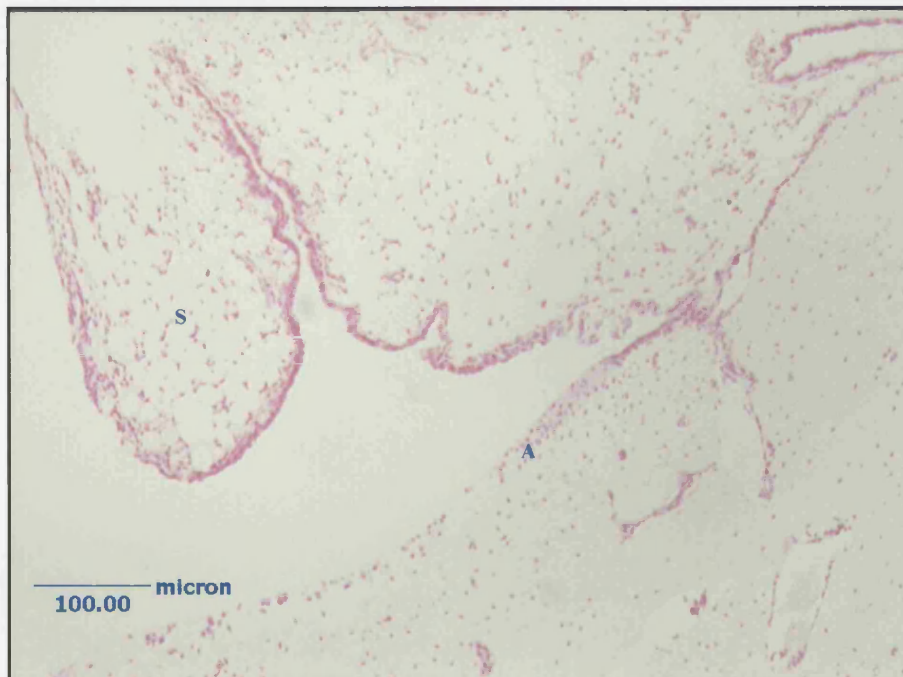


Plate 5.7 shows that staining for iNOS in the naïve hind feet is limited to the lining cells of the synovium and the smooth muscle of some but not all blood vessels. In the Adjuvant hind feet (plate 5.8) it can be seen that there is a very obvious increase in cells labelling for iNOS. The cells labelling for iNOS were in the pannus, the synovium and the leading edge of sites where bone erosion was occurring. Again areas of increased staining did not always specifically colocalize with areas of hypoxia.

Plate 6.9 shows that staining for VEGF in the naïve hind feet is limited to the inner most lining cells of the synovium and the endothelial cells of some but not all blood vessels. In the Adjuvant hind feet (plate 5.10) it can be seen that there is a very obvious increase in cells labelling for VEGF in areas that also demonstrate an increase in iNOS labelling. The cells labelling for VEGF were in the pannus, the synovium and the leading edge of sites where bone erosion was occurring. Again areas of increased staining did not always specifically colocalize with areas of hypoxia.

Plate 5.6 Adjuvant hind foot (x10, wax section, stained with mouse anti-rat ED1). Articular cartilage [A]. The stained cells extend beyond the lining cells of the synovium [S].

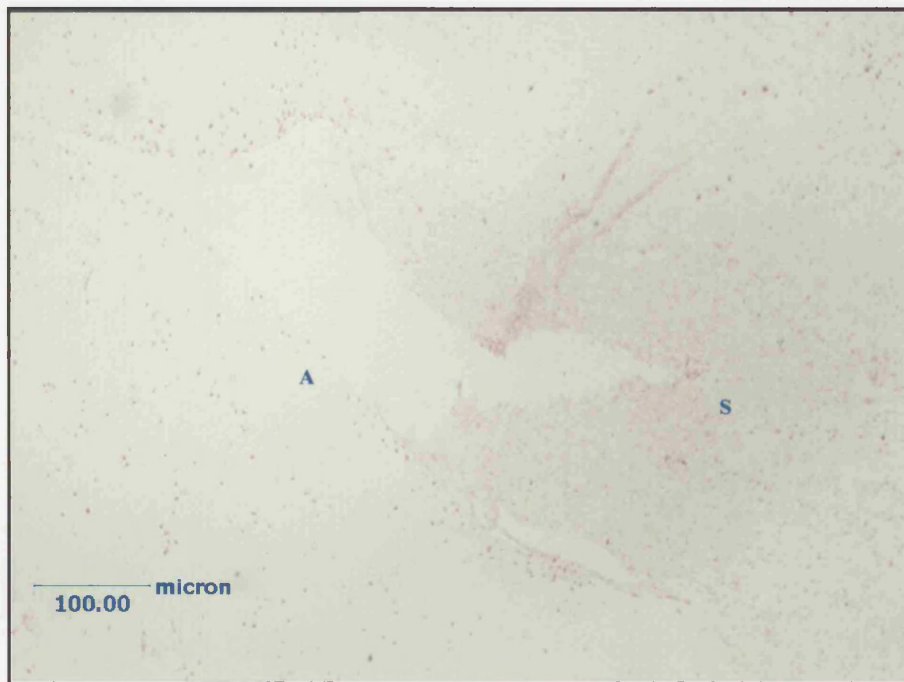


Plate 5.7 Adjuvant hind foot (x10, wax section, stained with mouse anti-rat ED2). Articular cartilage [A]. There are more stained cells and they extend beyond the lining cells of the synovium [S].

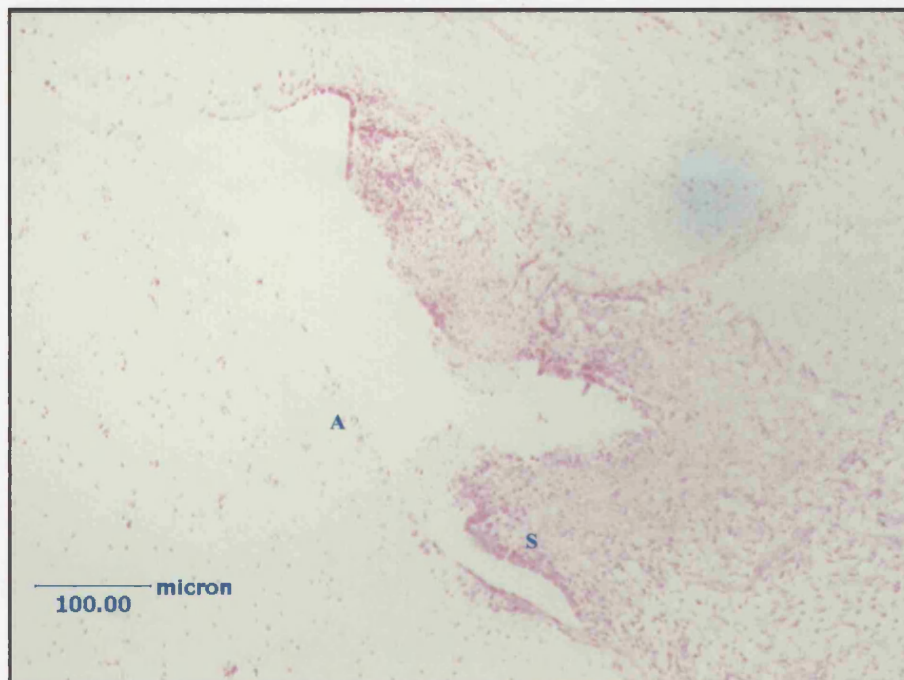


Plate 5.8 Naive hind foot joint (x10, wax section, stained with rabbit anti-iNOS). Staining of some blood vessel smooth muscle [BV] and lining cells of the synovium [S].

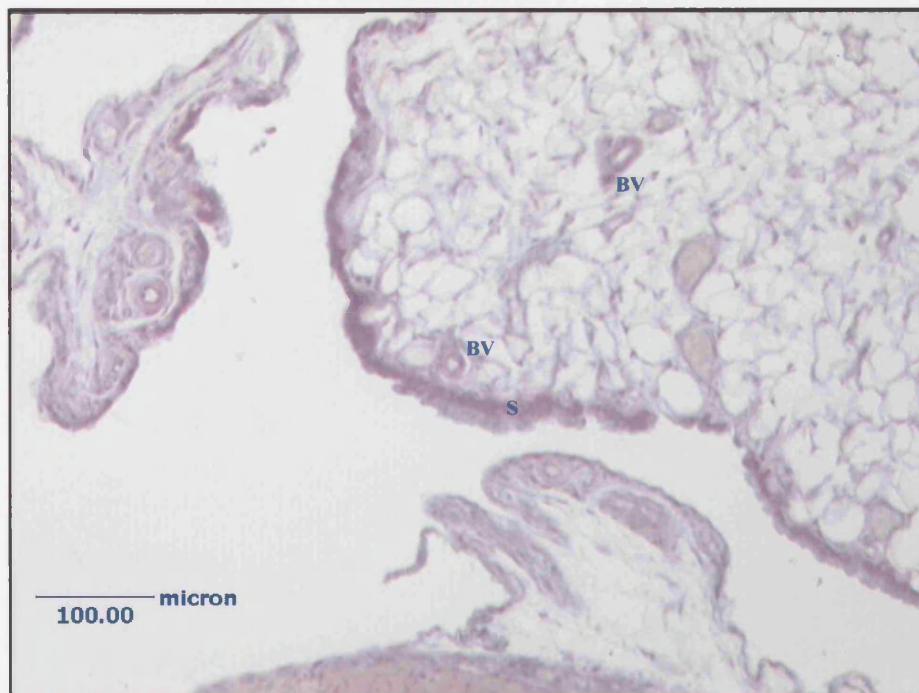


Plate 5.9 Adjuvant hind foot joint (x10, wax section, stained with rabbit anti-iNOS). Note heavy red staining in cells of the synovium [S] particularly at site of bone invasion [B].

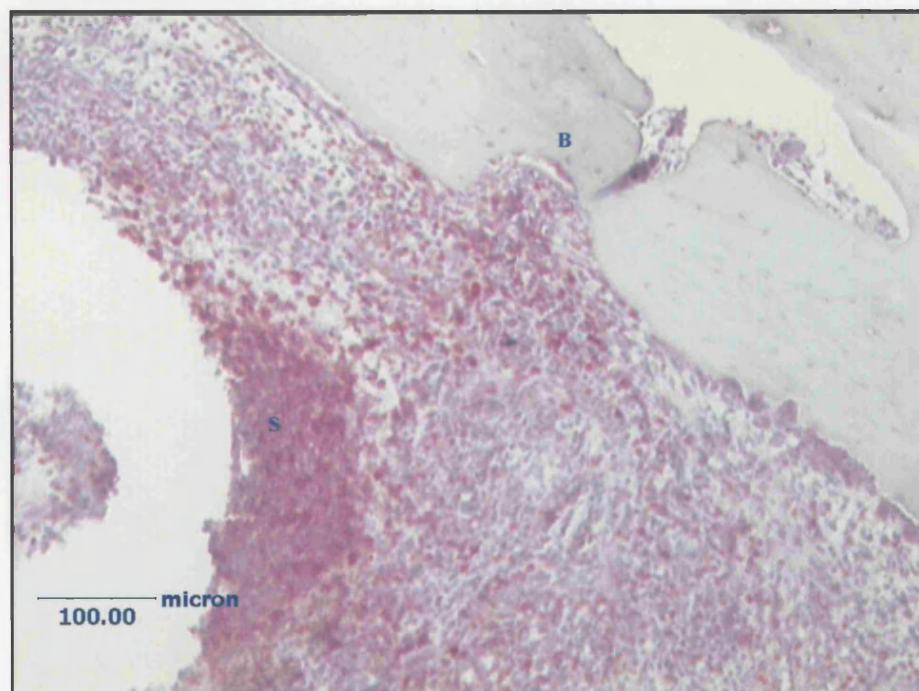


Plate 5.10 Naive hind foot joint (x10, wax section, stained with goat anti-VEGF). Staining of some blood vessel smooth muscle [BV] and lining cells of the synovium [S].

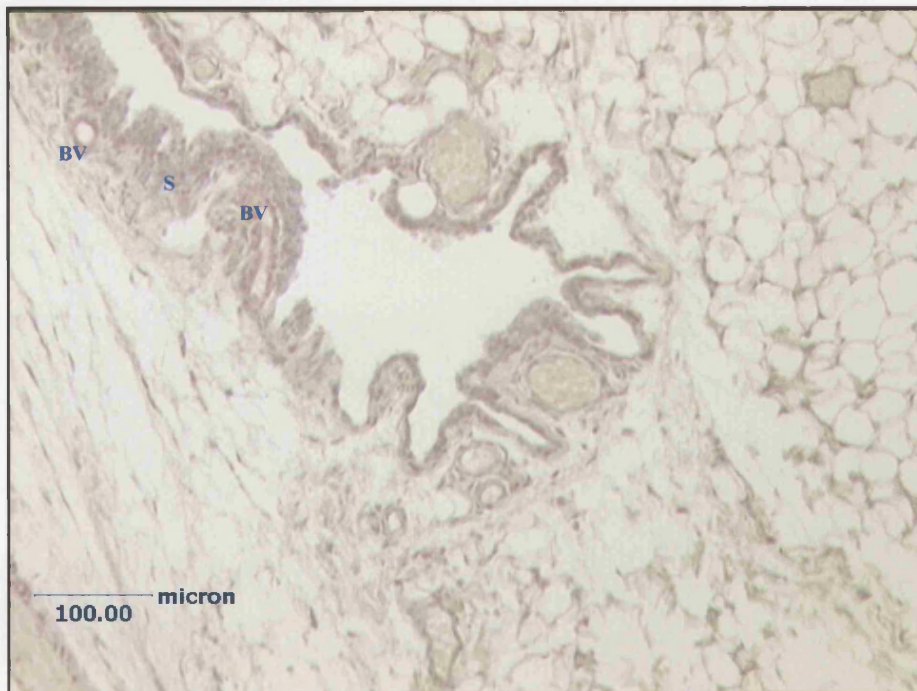


Plate 5.11 Adjuvant hind foot joint (x10, wax section, stained with goat anti-VEGF). Note heavy red staining in cells of the synovium [S] particularly at site of bone invasion [B].

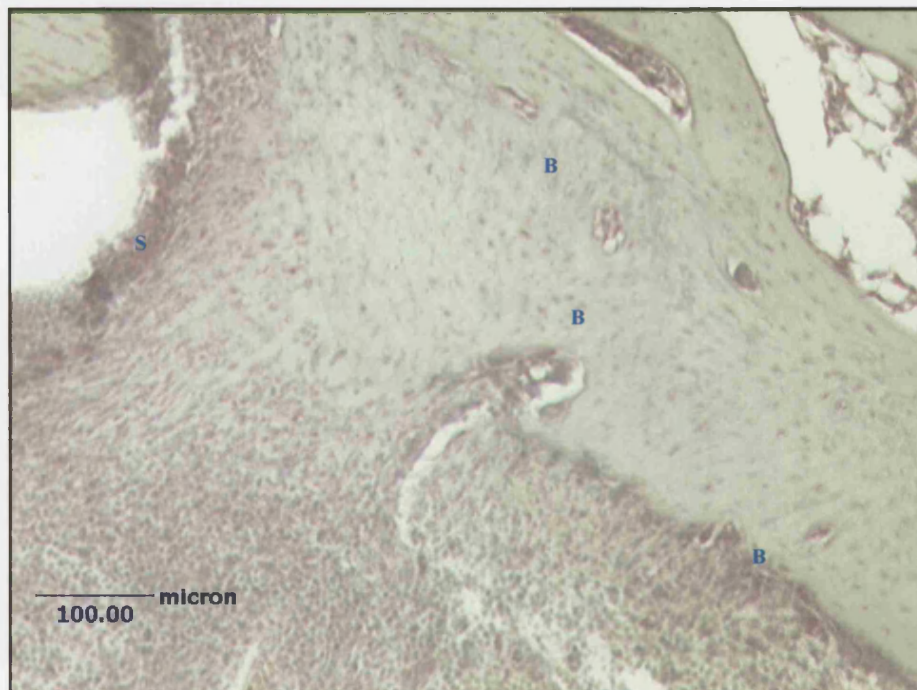


Plate 5.10 Naive hind foot joint (x10, wax section, stained with goat anti-VEGF). Staining of some blood vessel smooth muscle [BV] and lining cells of the synovium [S].

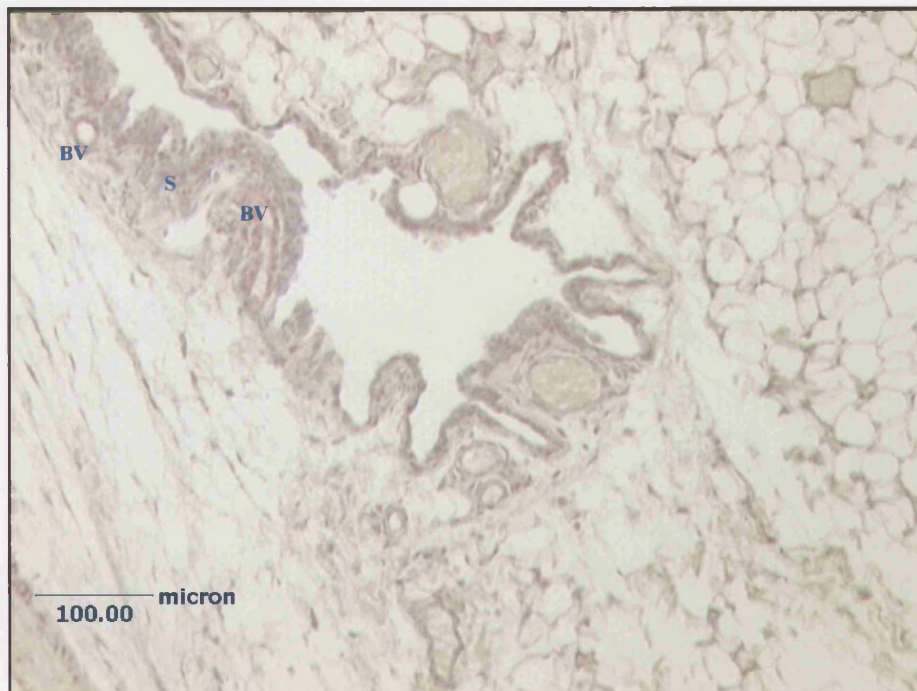
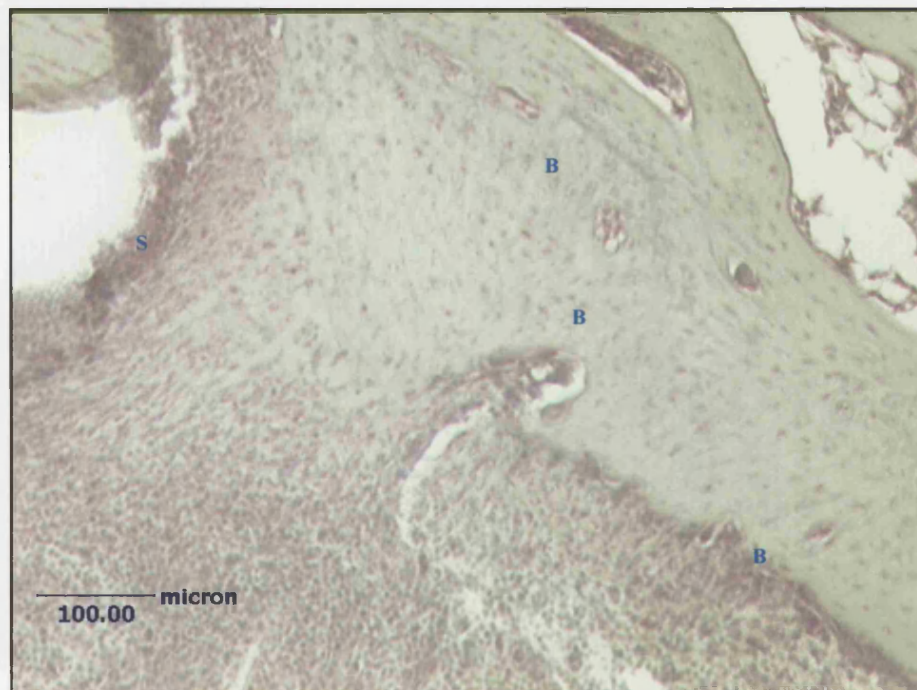


Plate 5.11 Adjuvant hind foot joint (x10, wax section, stained with goat anti-VEGF). Note heavy red staining in cells of the synovium [S] particularly at site of bone invasion [B].



Positive immunoreactivity (red reaction product) for Hypoxyprobe-1 adducts (1MAB1-ir) and Hypoxia inducible factor-1 α (HIF1 α -ir, a brown reaction product) in the adjuvant foot tissue involved all structures including the synovium, pannus, bone marrow and articular cartilage chondrocytes.

A representative set of stained sections presented in Plates. 5.12 and 5.14 show that cell nuclei with positive immunoreactivity for ets-1 (Ets1-ir - red reaction product) are mainly found in the inflammatory infiltrate and invasive pannus of the inflamed synovial joint. There is co-localization with areas of positive 1MAB1-ir in flip-flop sections (Plates. 5.13 and 5.15). The histologically normal hind feet from naïve rats showed a low level of ets-1-ir in the sub-intimal synoviocytes (Plates 5.16 and insert), that did not co-localise with 1MAB1-ir (Plates. 5.17 and insert).

A representative set of immunohistochemical sections is presented in Plates. 5.18 and 5.20 showing cell nuclei with positive immunoreactivity for Ets1-ir mainly in the inflammatory infiltrate and the invasive pannus of the inflamed synovial joint colocalizing with nuclei with strong positive HIF1 α -ir in flip-flop sections (Plates. 5.19 and 5.21). These sections are 5 μ m thick alternate downward and upward facing (flip-flop) therefore the stained cells are the same cells in both Plates. 5.20 and 5.21. The hind feet from naïve rats showed a low level of ets-1-ir in the sub-intimal synoviocytes (Plates. 5.22 and insert), that did not co-localise with HIF1 α -ir (Plates. 5.23 and insert).

Plate 5.12 Adjuvant hind foot joint (x10, wax section, stained with rabbit anti-ets1). Red stain for ets-1 in blood vessel [BV] and inflammatory infiltrate. Co-localizing with hypoxyprobe adducts in plate 6.13

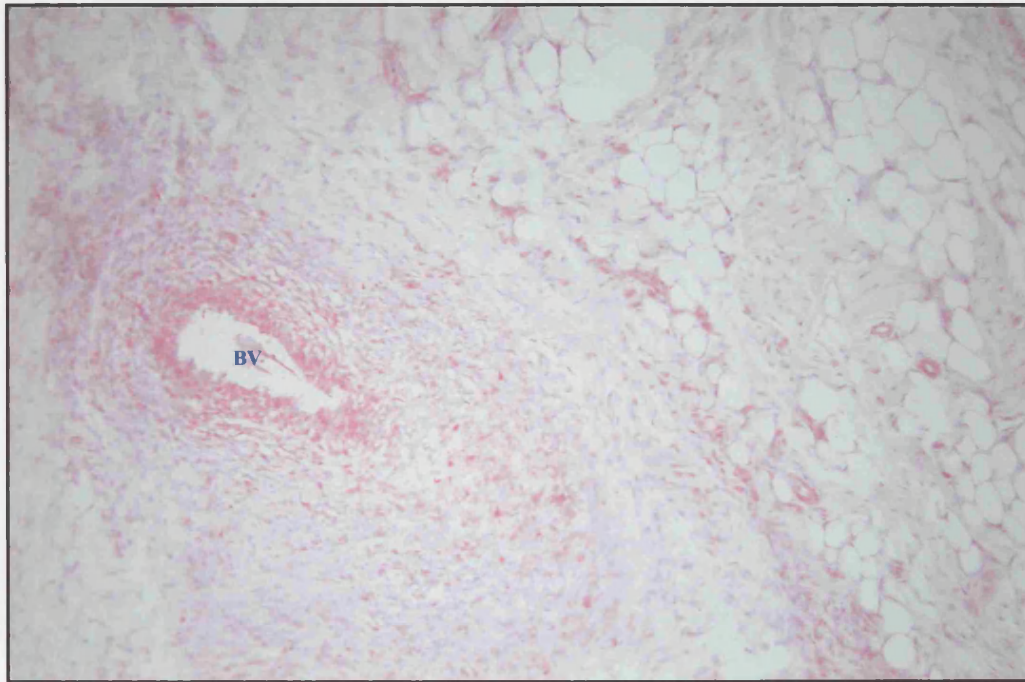


Plate 5.13 Adjuvant hind foot joint (x10, wax section, stained with mouse anti-Hypoxyprobe). Red stain in blood vessel [BV] and inflammatory infiltrate.

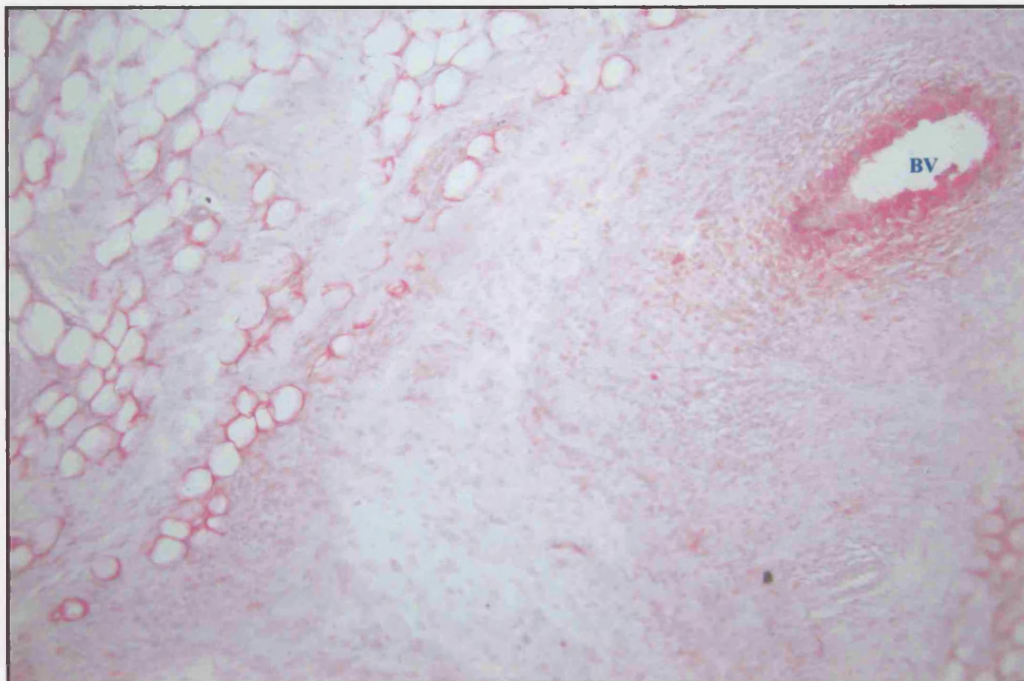


Plate 5.14 Adjuvant hind foot joint (x10, wax section, stained with rabbit anti-ets1). Red nuclear stain for ets-1 in proliferating cells of synovium. Co-localizing with hypoxyprobe adducts in plate 6.15

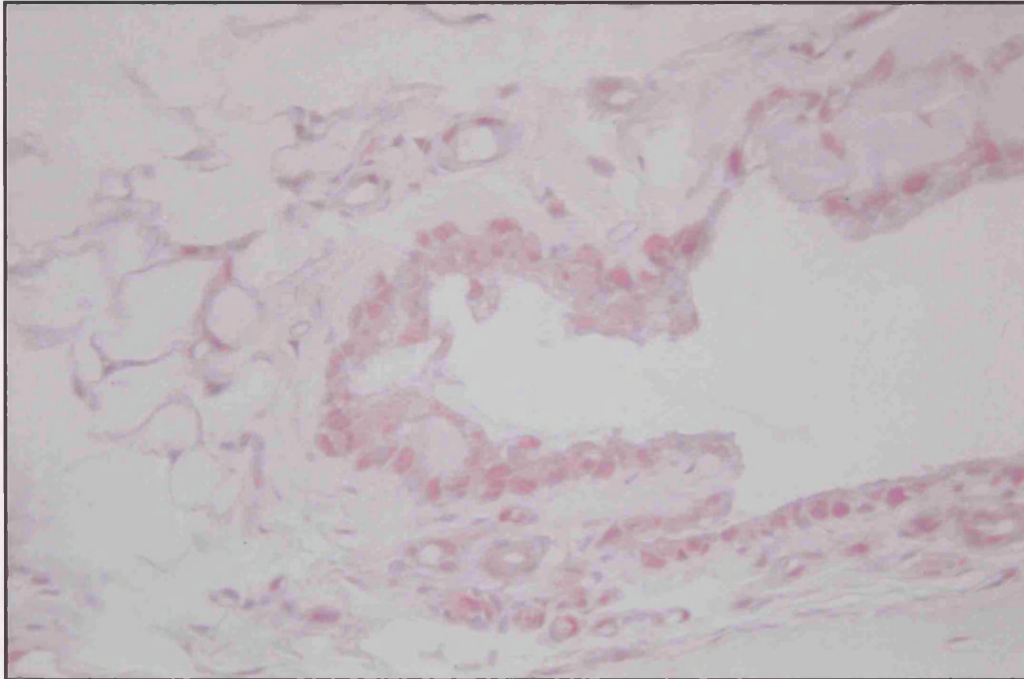


Plate 5.15 Adjuvant hind foot joint (x10, wax section, stained with mouse anti-Hypoxyprobe). Red stain in proliferating cells of synovium.

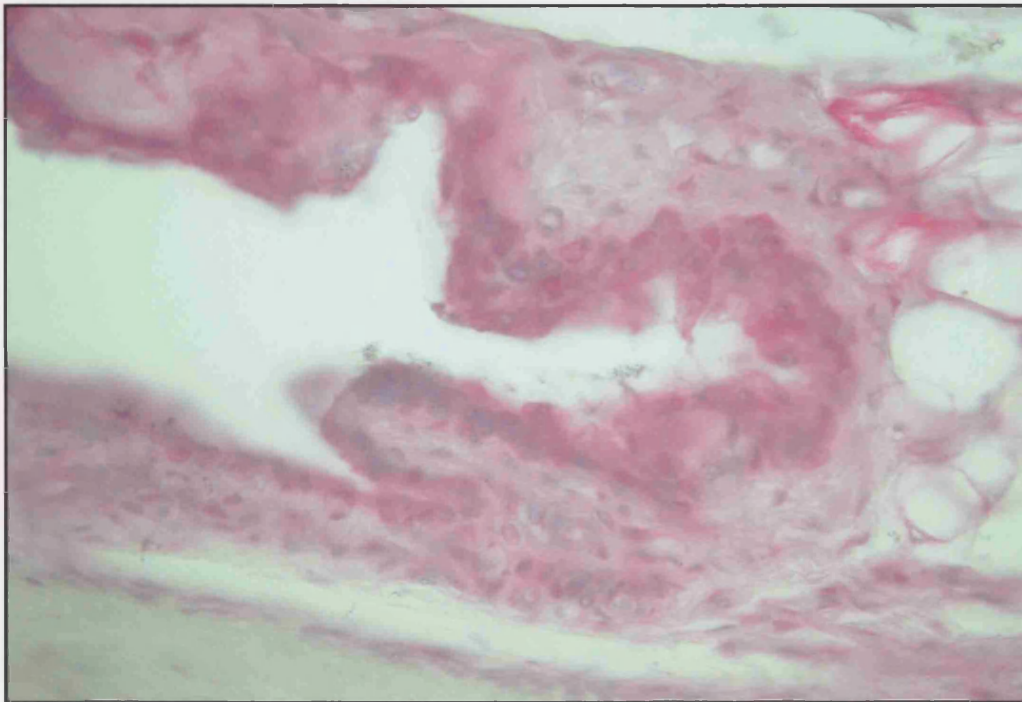


Plate 5.16 Naive hind foot joint x40, wax section, stained with rabbit anti-ets1(insert x10 of same section). Low level, staining for ets-1.

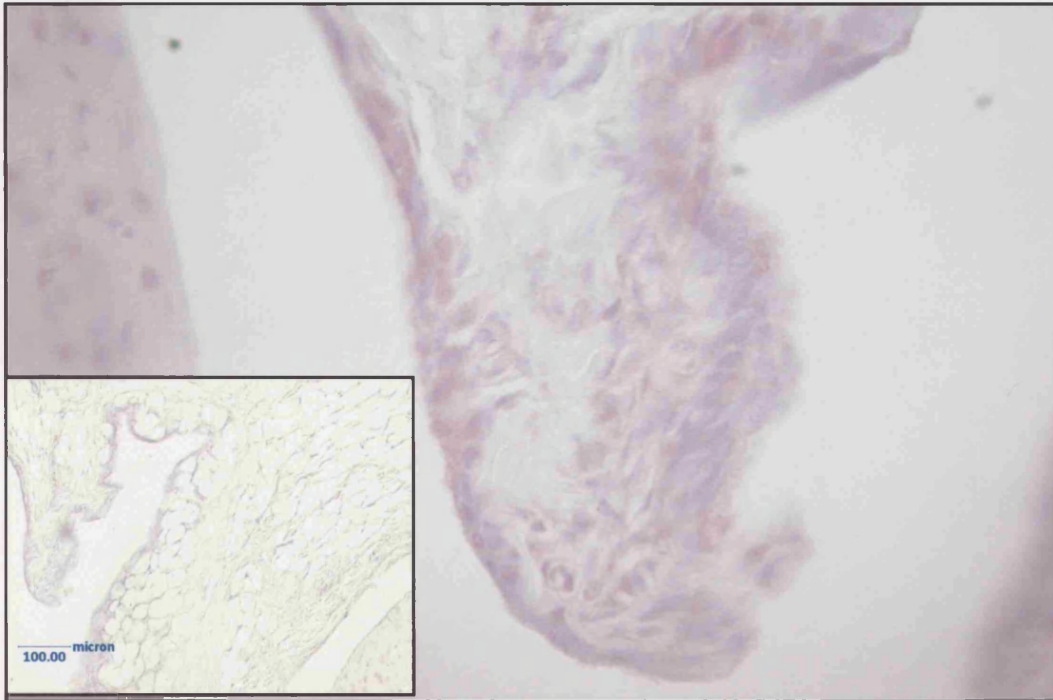


Plate 5.17 Naive hind foot joint x40, wax section, stained with mouse anti-hypoxypore (insert x10 of same section). Low level, non nuclear staining, no co-localization with plate 6.16

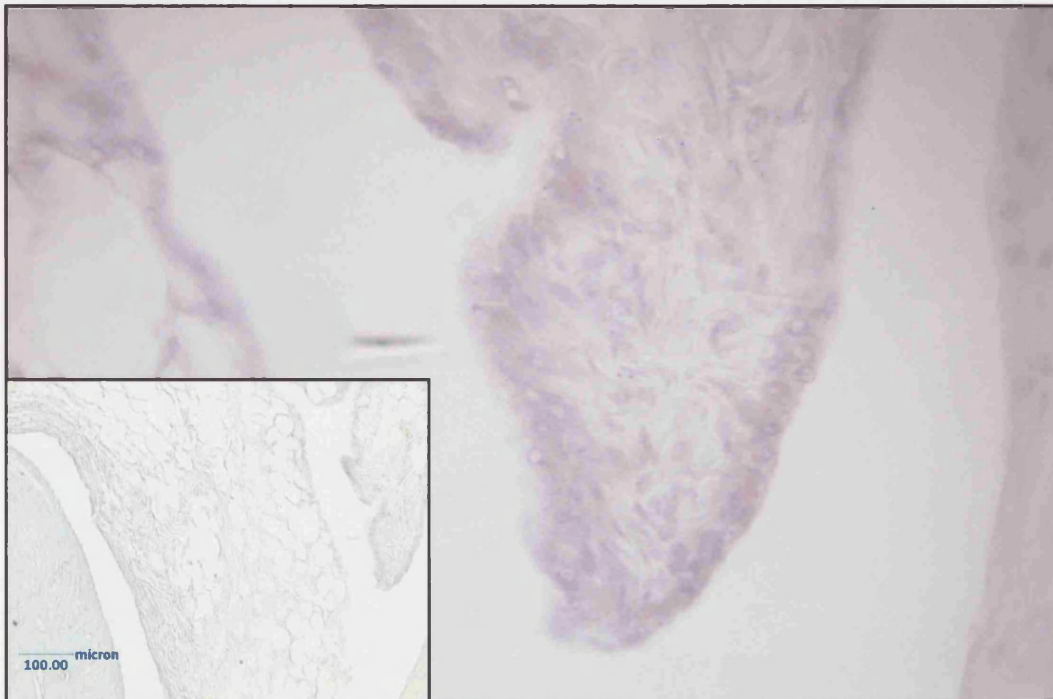


Plate 5.18 Adjuvant hind foot joint (x10, wax section, stained with rabbit anti-ets1). Red stain for ets-1 in blood vessels [BV] and lining cells of the proliferating synovium [S]. Co-localizing with HIF-1 α in plate 6.19

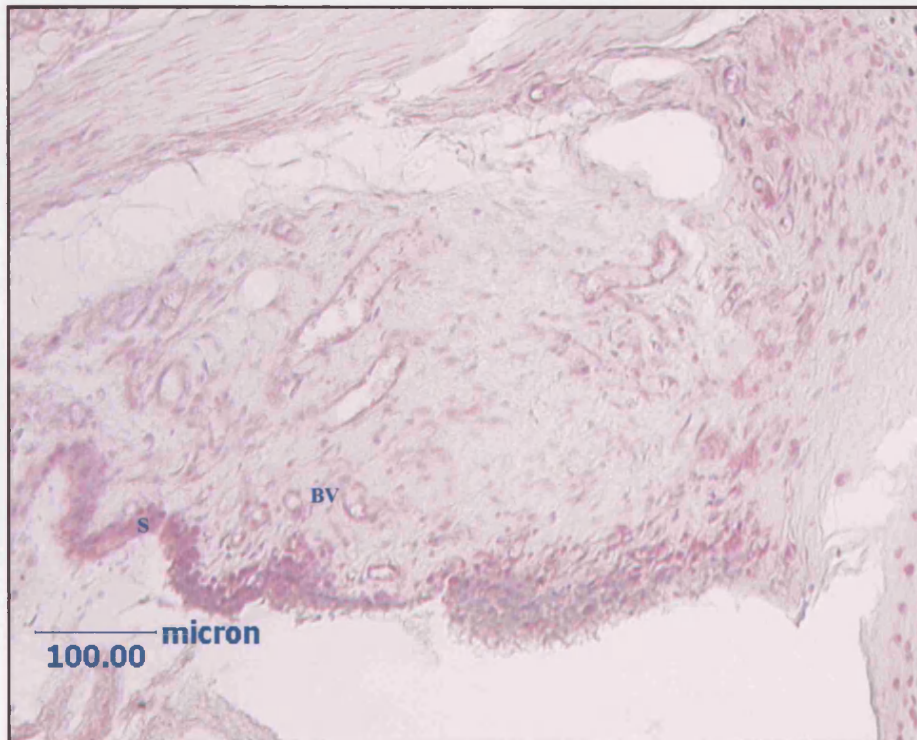


Plate 5.19 Adjuvant hind foot joint (x10, wax section, stained with mouse anti-HIF-1 α). Brown stain for HIF-1 α in blood vessels [BV] and lining cells of the proliferating synovium [S].

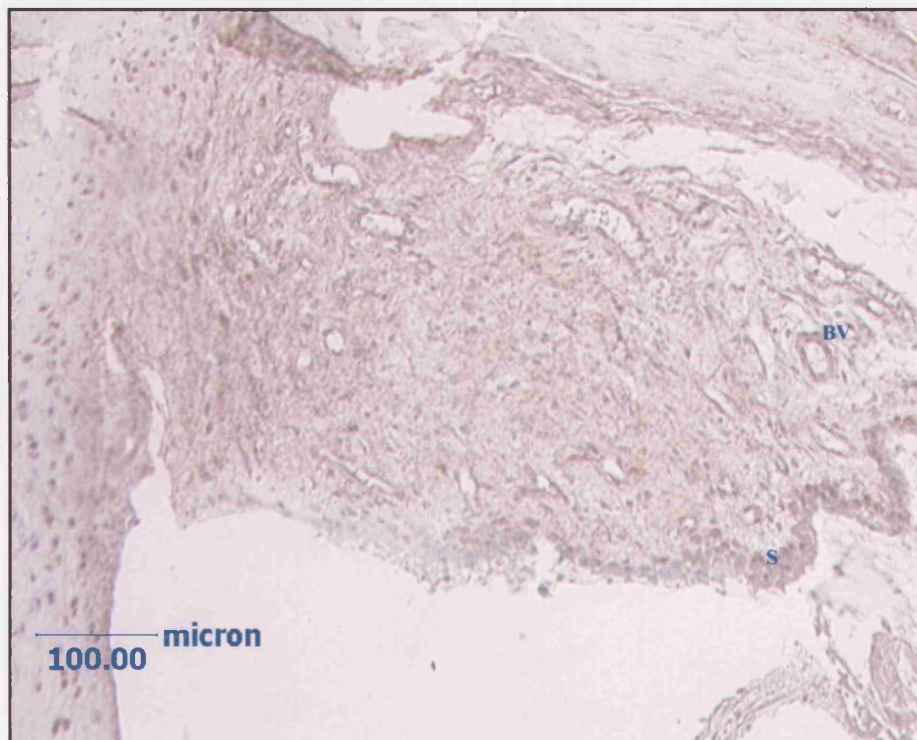


Plate 5.20 Adjuvant hind foot joint (x40, wax section, stained with rabbit anti-ets1). Red nuclear stain for ets-1[arrows] in nuclei of lining cells of the proliferating synovium. Co-localizing with HIF-1 α in plate 6.21

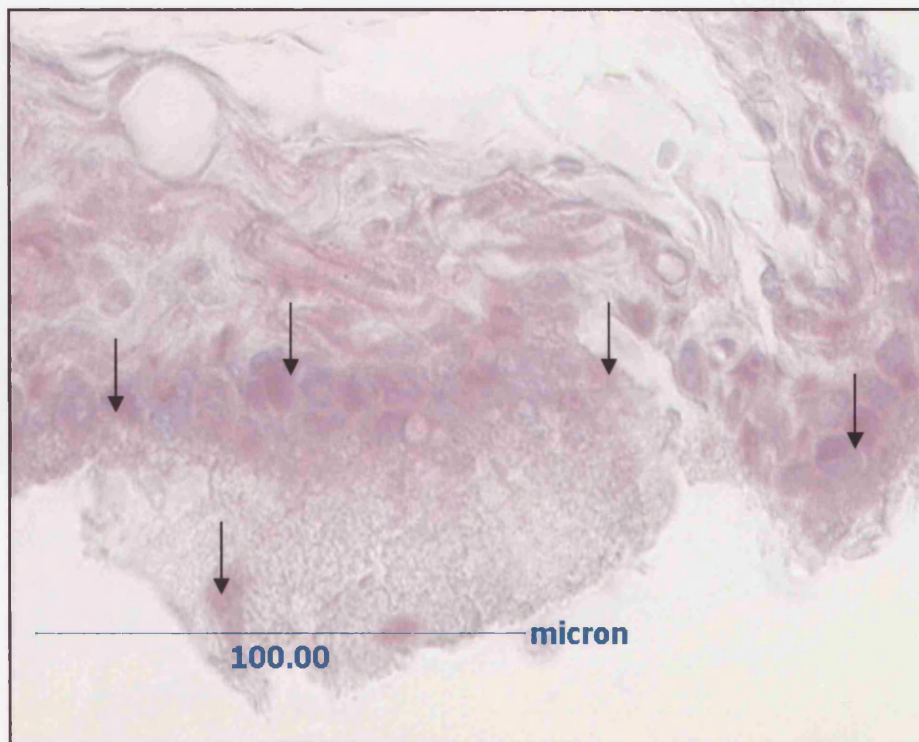


Plate 5.21 Adjuvant hind foot joint (x40, wax section, stained with mouse anti-HIF-1 α). Brown nuclear stain for HIF-1 α [arrows] in nuclei of lining cells of the proliferating synovium. Co-localizing with ets-1 α in plate 6.20.

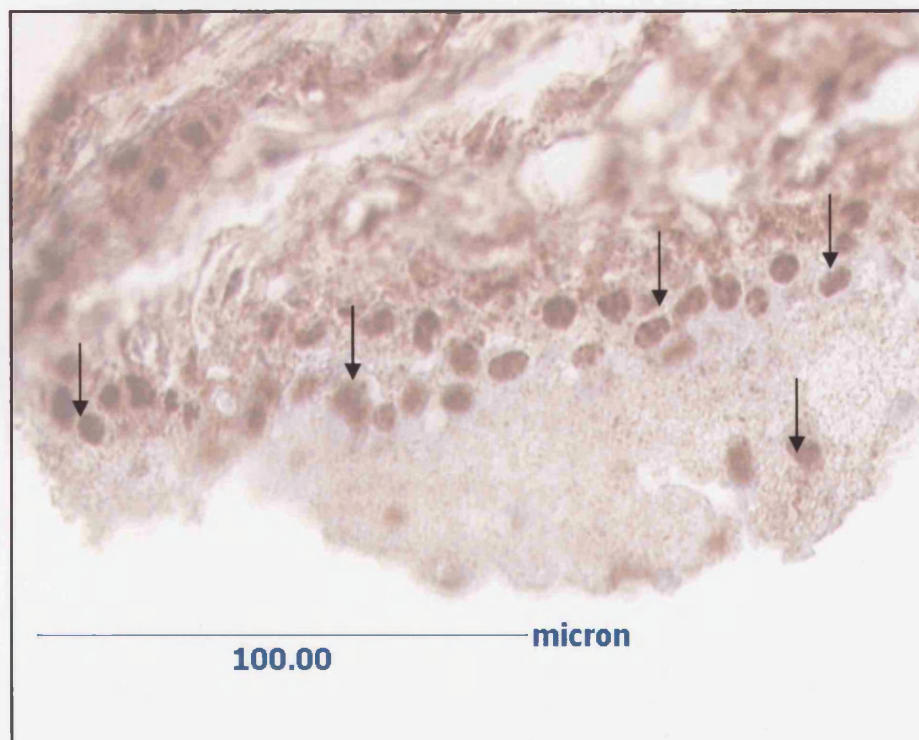


Plate 5.22 Naive hind foot joint x40, wax section, stained with rabbit anti-ets1(insert x10 of same section). Low level, staining for ets-1.

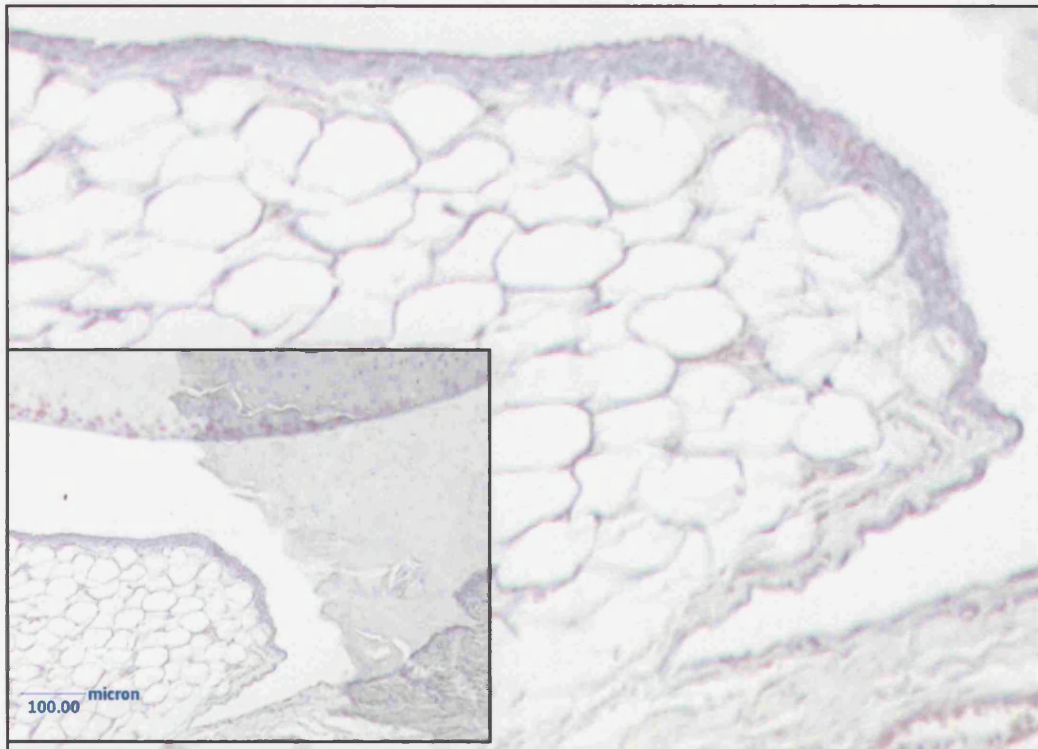
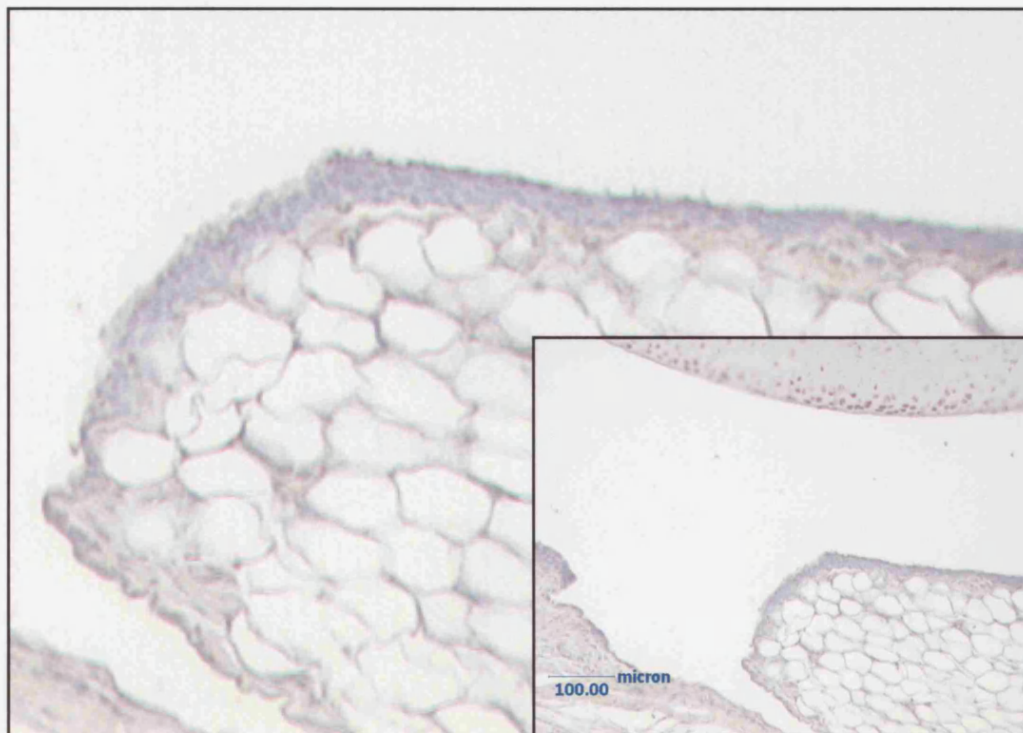


Plate 5.23 Naive hind foot joint x40, wax section, stained with mouse anti-HIF-1 α (insert x10 of same section). Low level, no nuclear staining for HIF-1 α



5.3.2.3 Quantitative Assessment of ets-1 and HIF-1 α immunohistochemistry

The hind feet from adjuvant rats showed a highly significant ($p < 0.0001$) increase in the proportion of cells with positive nuclear ets-1 immunoreactivity (Ets1-ir) compared to naïve control rats (Figure 13). They also showed a significant ($p < 0.0001$) increase in hypoxic tissue as demonstrated by positive 1MAB1-ir compared to naïve control rats (Figure 14). Figure 15 shows that the hind feet from adjuvant rats showed a highly significant ($p < 0.0001$) increase in the proportion of cells with positive nuclear Ets1-ir that also colocalized with HIF1 α -ir compared to naïve control rats. Statistical comparisons were made using the Mann-Whitney Test.

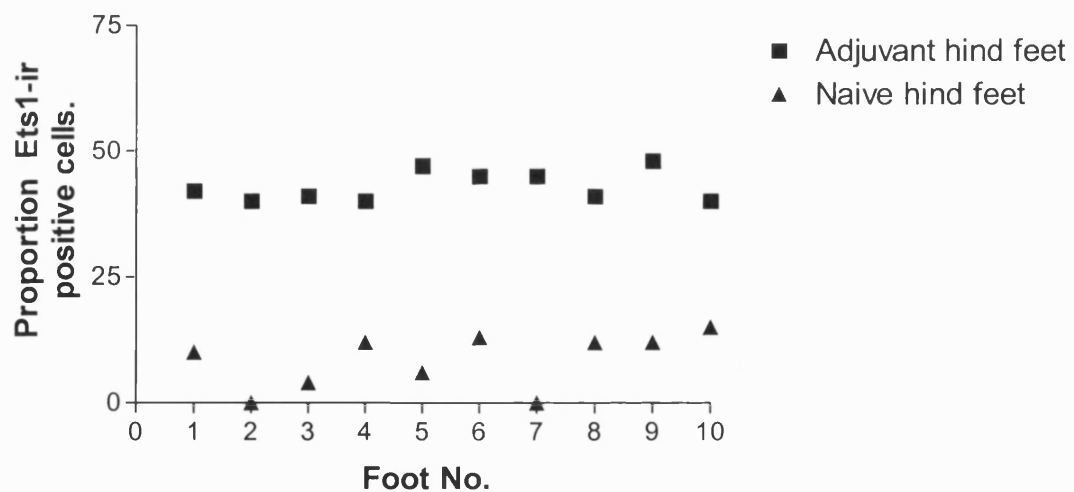


Figure 13. Proportion of nuclei with positive Ets- immunoreactivity.

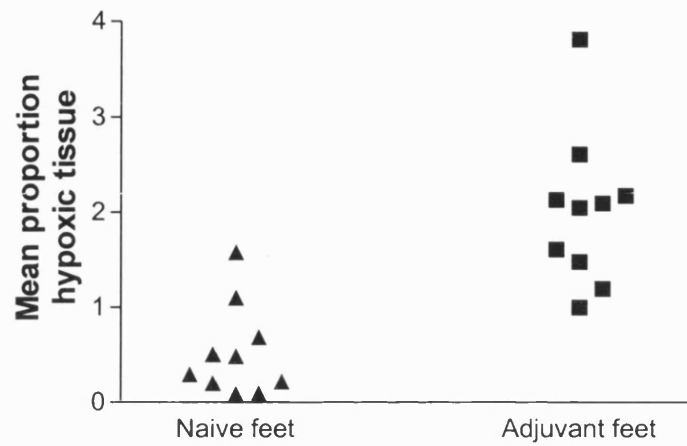


Figure 14. Proportion of hypoxic tissue.

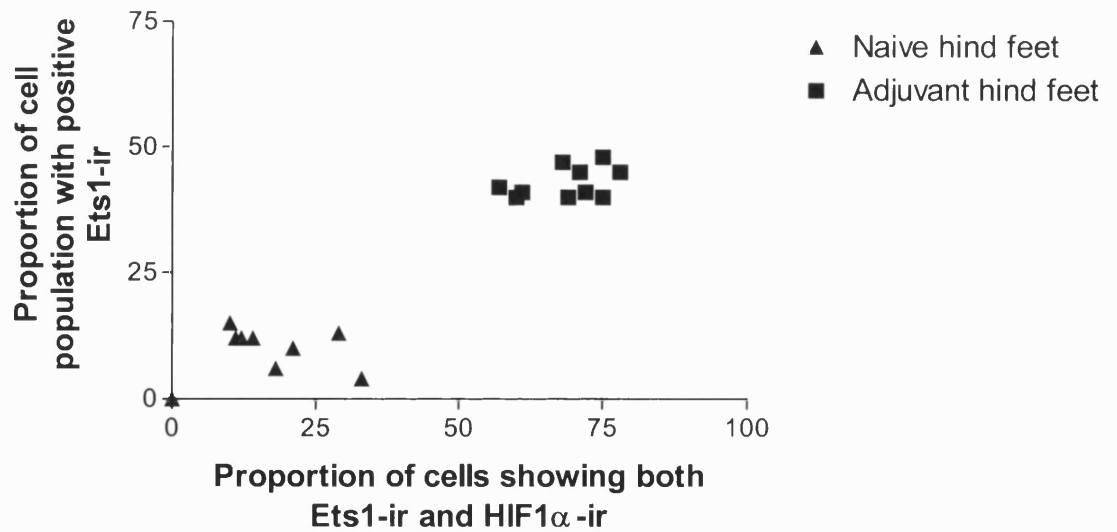


Figure 15. Proportion of nuclei HIF1 α -ir colocalizing with ets-1-ir.

5.4 Discussion

These results confirm that inflamed joints in the rat adjuvant model possess significantly more hypoxic tissue, as demonstrated by positive hypoxyprobe-1 adduct immunoreactivity, than those from naïve controls. This concurs with the situation in the rheumatoid joint (Blake et al., 1997). However, the studies in this chapter have not identified any phenotypic specific marker associated with the cells labelling with hypoxyprobe seen in chapter 4.

There was also a clear increase in numbers of cells expressing ED2 and to lesser extent ED1 macrophage markers showing that cells proliferate and are recruited from the circulatory system to the site of inflammation. However, the increase in ED1 and ED2 expressing cells did not involve co-localizations with hypoxyprobe adduct labelling. This indicates that it is likely to be other factors, cytokines for example, that play the key role in the expansion of ED1 and ED2 cell populations.

The fibroblast marker did not stain anything, this is probably because this antibody although generated against a peptide that shows apparent homology in humans and rats, is essentially derived from humans and shows no rat cross reactivity in my studies. This is probably also the reason for the lack of staining observed for the VCAM-1 antibody. I would expect both these markers to be present in cells in the inflamed joint. As discussed in chapter 1.3.2, the cells in the synovium possess characteristics that delineate them as macrophage-like or fibroblast-like synoviocytes. VCAM-1 would also be expected to be increased as it plays a key role in the trafficking of leukocytes from the circulatory system to the site of inflammation and increased VCAM-1 expression has been demonstrated in the

synovium from RA patients (Morales-Ducret et al., 1992). Fresh frozen synovial tissue, generally did not demonstrate specific immunoreactivity. This was probably due to the lack of protein cross linking in the fixation technique which relied only on acetone to fix the proteins. Better antigenicity might have been achieved had the samples been perfusion fixed prior to snap freezing. It is highly probable, however, that the hypoxic cells identified in the inflamed joint are a heterogeneous population with phenotypical characteristics from both fibroblast and macrophage lineages.

My results did show a qualitative increase in both iNOS and VEGF expressing cells and these results mirror data previously published that demonstrate an increase in iNOS (Sakurai et al., 1995, Gabowski et al., 1997) and VEGF (Jackson et al., 1997) in the rheumatoid synovium. I believe that the localisation of these two proinflammatory factors in areas where bone erosion was taking place is indicative of the fact that they play a key role in this process. Neither iNOS nor VEGF expression colocalized with areas of hypoxia as indicated by hypoxyprobe labelling, suggesting that hypoxia does not play a critical role in their expression. I speculate that initiating factors are likely to be cytokines like TNF α and IL-1. These results support recently published work showing that an increase of the VEGF/KDR (kinase insert domain protein receptor) pathway occurs independent of increases in HIF-alpha expression in rheumatoid arthritis (Giatromanolaki et al., 2003). This disparity between hypoxia and cytokine expression is also reflected in other situations. Haroon et al (2000) used Hypoxyprobe-1 to demonstrated that early wound healing in a rat model exhibits a cytokine surge without evidence of hypoxia. Their results showed that hypoxia peaked in the granulation tissue and correlated with increased cellular proliferation. In a study of human squamous cell carcinomas tumour Hypoxyprobe-1 was used in a

immunohistochemical comparison of hypoxia and VEGF protein expression and revealed no correlation between the two (Raleigh et al., 1998).

In my study immunohistochemical localization of hypoxia inducible factor-1 α involved all structures including the synovium, pannus, bone marrow and articular cartilage chondrocytes of the adjuvant foot tissue and this mirrors my work from chapter 5 using hypoxyprobe. This contrasts with work carried out by Hollander et al., (2001), who demonstrated that HIF-1 α localised specifically to macrophages. My results are, however, comparable to similar studies carried out more recently in rheumatoid synovium. Two groups Hitchon et al., (2002) and Giatromanolaki et al., (2003) have published immunohistochemical studies that localized HIF-1 α in synoviocytes lining the inflamed synovial tissue. Their images were a mirror of my images of HIF-1 α in adjuvant inflamed synovium.

The ets-1 promoter has been shown to contain hypoxic responsive like elements (Oikawa et al., 2001) and I expected increased cellular hypoxia to be associated with a concurrent increase in the expression of ets-1. This was verified by my results showing that in joints from all ten feet from animals with adjuvant arthritis a minimum of 40 percent of nuclei in the total cell population were positive for ets-1, compared to the ten naïve joint samples in which no more than 15 percent expressed detectable ets-1. Furthermore, 65-75 percent of nuclei expressing ets-1 in the adjuvant samples also expressed HIF-1 α . This clearly demonstrates an induction of hypoxia and expression of ets-1 during the inflammatory response in adjuvant rat joints. That Ets-1 and HIF-1 α co-localize in areas of hypoxia in inflamed synovial tissue, suggests that both hypoxia and HIF-1 α may be involved in the upregulation of ets-1 during joint inflammation.

Others have demonstrated that ets-1 transcription factor up-regulation correlates with tumour invasion (Vandenbunder et al 1994, Wernert et al., 1994). Since I carried out this study, the same group have also identified ets-1 in the endothelium of synovial blood vessels (Wernert et al., 2002), however they made no observation of ets-1 in the pannus, probably because their research is primarily orientated to angiogenesis in tumour biology. As well as showing ets-1 expression in the blood vessel endothelium, my results also localize ets-1 in cells on the edge of the invasive pannus. These are clearly hypoxic, as indicated by both hypoxyprobe labelling and HIF-1 α expression and are distant from blood vessels. Ets-1 expression would be expected to be present in the endothelium if the blood vessels of the synovium were promoting angiogenic messages. Indeed, as discussed in the introduction (1.6), there is accumulating evidence to show that pro-angiogenic signals and abnormal blood vessel formation has been associated with rheumatoid arthritis (Walsh et al., 1999). That Ets-1 and HIF-1 α co-localize in areas of hypoxia in inflamed synovial tissue, suggests that both hypoxia and HIF-1 α may be involved in the upregulation of ets-1 during joint inflammation.

The localisation of ets-1 in the invasive pannus is indicative of ets-1 involvement in the invasion of synovial joint cartilage and bone by proliferating synovium. This theory is further supported when you consider the broad range of target genes for ets-1. This has been extensively reviewed (Lelievre et al., 2001), areas of particular relevance to the pathology of arthritis being its involvement in angiogenesis and extracellular matrix remodelling. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) such as collagenase 1 (MMP-1), stromelysin (MMP-3), gelatinase B (MMP-9) and TIMP-1 are all gene targets of ets-1 and have been shown to be elevated in patients with

RA (Vincenti et al., 1994 and Klimiuk et al., 2002). Indeed more recent work by Czuwara-Ladykowska (2002) has shown that elevated ets1 expression in fibroblasts alters their response to TGF β in favour of matrix degradation. These results could implicate ets-1 as a key player in the destruction of the bone and cartilage in rheumatoid arthritis by fibroblast like synoviocytes and independent of its role in angiogenesis. I propose that hypoxia in the inflamed rheumatoid joint results in increased HIF-1 α expression; HIF-1 α binds to hypoxic response like binding sequences on the ets-1 promoter which leads to increased expression of ets1 by fibroblast like synoviocytes. This throws a switch in the direction of matrix degradation and thus the destruction of the joint.

CHAPTER 6: Effect of Induced Hypoxia on Transcription Factors *Invitro*

6.1 Introduction

A distinctive characteristic of the rheumatoid joint is rapid synovial proliferation which outstrips neovascularization (Stevens et al., 1991). This may result in metabolic hypoxia in proliferating cells and it has been shown by oxygen electrode studies that low oxygen tensions (<10mm/Hg) do exist in the rheumatoid joint cavity (Blake et al., 1997). Hypoxia is a potent inducer of cytokines, matrix degrading enzymes and angiogenic factors and has a central role in the normal inflammatory response. Moreover, hypoxia-driven angiogenesis is critical for tissue remodelling (Steinbrech et al., 1999). Critically, cartilage and bone are invaded by the proliferating synovium (pannus) resulting in erosion and joint destruction. The reason for this invasiveness is not understood but in tumours, invasiveness is marked by upregulation of the ets-1 transcription factor (Wernert. 1994). Ets-1 is particularly relevant to the rheumatoid joint as it can be induced during hypoxia by activation of a second transcription factor, hypoxia-inducible factor 1 (HIF-1).

In chapter 5, I demonstrated the presence of Hypoxia and ets-1 expression in the inflamed joint of an animal model. The objective of this chapter is to validate and support the *in vivo* studies by the use of an *in vitro* system to expose synoviocytes to hypoxia and compare them to normoxic cells. ets-1 and HIF-1 expression is determined *in vitro*, in hypoxic rheumatoid synovial fibroblasts (RSF) and hypoxic adjuvant rat synovium.

6.2 Materials and Methods

Adjuvant arthritis was induced in female Lewis rats 130-150g (n=10) using methods described in chapter 2.1.5. A second group of non-injected rats (n=10) served as naive controls. Disease progression was monitored daily using a standard clinical scoring system described in 2.1.6. Animals were killed by cervical dislocation on reaching a maximum clinical score of 4 in two hind feet. Naive rats were sacrificed at the same time. The knee patella suspensory ligaments were dissected and used in an explant culture technique developed within the School of Health at the University of Bath and described in chapter 2.4.6. Synovial fibroblasts from rheumatoid arthritis patients were obtained with patient's consent according to local ethical committee procedures and kindly donated by Dr. D. Speden. The cells used for experiments were passage 1-5 and had been checked for fibroblast phenotype using specific antibodies Mouse Monoclonal Anti Fibroblast 5B5 clone (DAKO, Ely, UK) via immunocytochemistry by Dr. D. Speden. The cells were cultured by methods described in chapter 2.4. A microflow anaerobic System (M.D.H Ltd., Hampshire, UK.) was used to incubate the cells and explants under quantifiable hypoxic conditions ($pO_2 < 10$ mm Hg at 37°C) for two weeks and compared to matched normoxic controls (method described in chapter 2.5). The culture media was then removed and frozen at -70°C for future studies. Explants were fixed and embedded for histological studies (methods described chapter 2.2). Protein was isolated from the cells by treatment with 2ml RNAase (AMS Biotechnologies, Oxford, UK) which was then stored in -70°C till use. The ets-1 and HIF-1 α protein was measured using SDS-PAGE on 7.5% gels and Western blotting with chemiluminescence methods described in chapter 2.6 to 2.8.6. The antibodies were those used previously in immunohistochemistry for the expression of

transcription factor ets-1 and HIF-1 α (see 2.2.4). JURKAT, rat kidney explant (DAKO, UK) and HeLa cells (section 2.4.2) were used as positive controls and negative control primary antibodies mouse monoclonal anti-*Aspergillus niger* glucose oxidase and rabbit IgG (section 2.2.4) were also used. Blots were analyzed by scanning densitometry (Scion Image Beta 4.02 - Scion Corporation, Maryland, USA.)

6.3 Results

6.3.1 Effect of hypoxia on ets-1 and HIF-1 α protein expression in synovial fibroblasts from rheumatoid patients (RSF).

Having demonstrated the presence of hypoxia and ets-1 expression in the inflamed joint of an animal model an *in vitro* system was utilized to expose synovial fibroblasts from rheumatoid arthritis patients to hypoxia and compare them to normoxic cells. I expected an increase in HIF-1 α protein expression in RA synovial fibroblasts exposed to hypoxia compared to normoxic fibroblasts; my results in figure 16a,b showed this. Figure 17a,b shows the concurrent increase in ets-1 protein expression in hypoxic RA synovial fibroblasts compared to normoxic synovial fibroblasts.

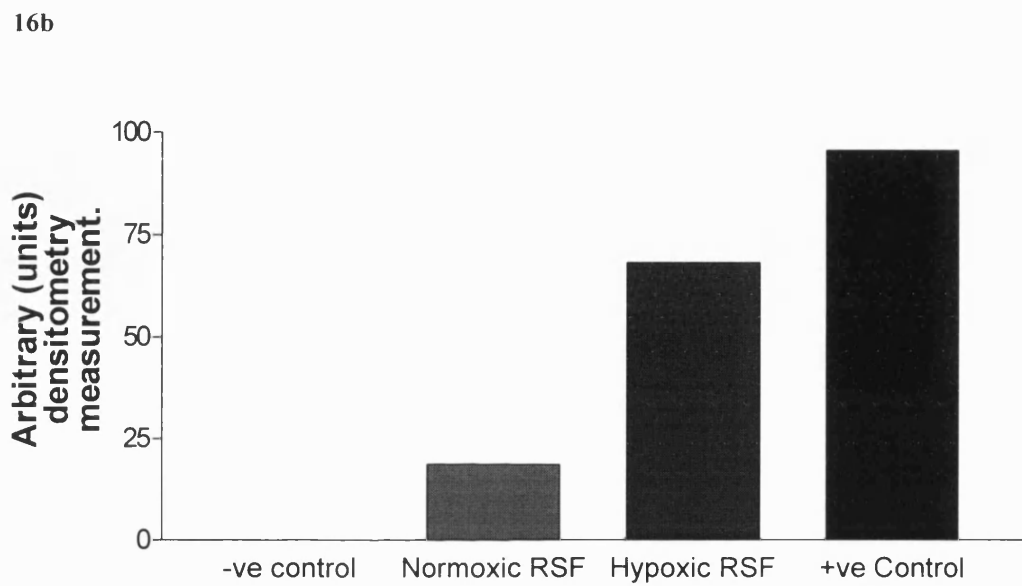
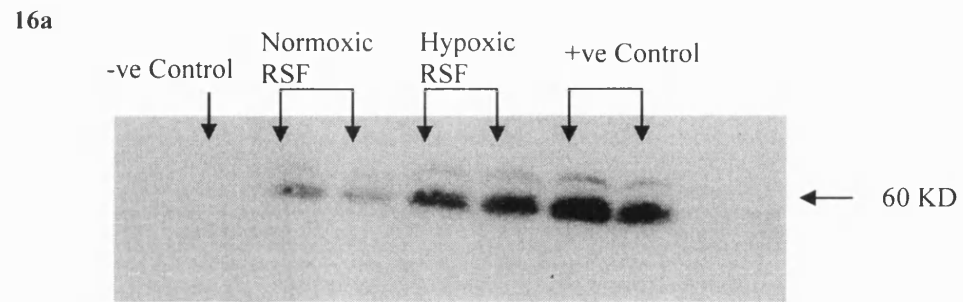


Figure 16a. Western Blot: Effect of hypoxia on HIF-1 α protein expression.

Figure 16b. Effect of hypoxia on HIF-1 α protein expression densitometry measurement.

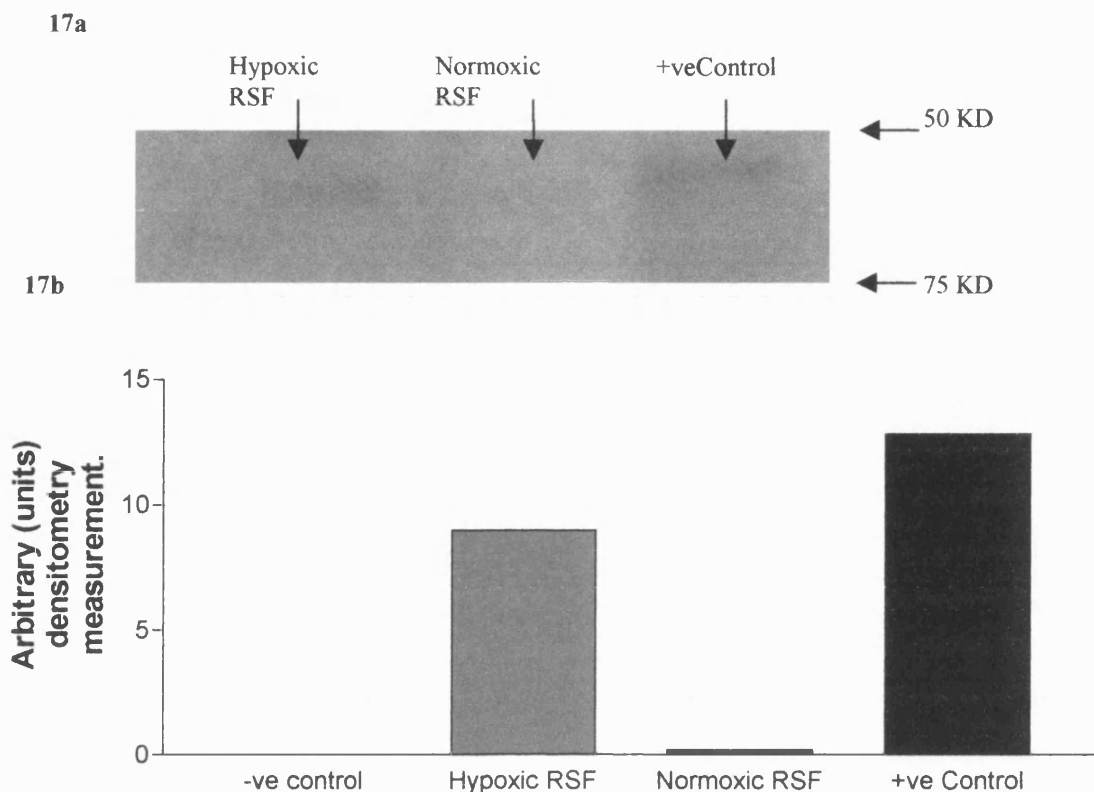
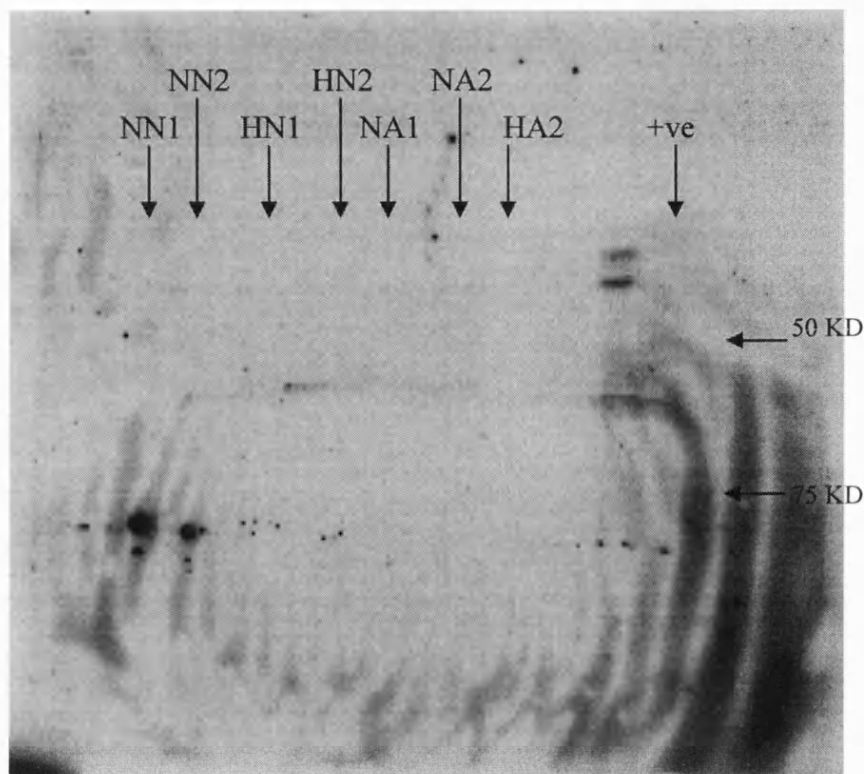


Figure 17a. Western Blot: Effect of hypoxia on ets-1 protein expression.

Figure 17b. Effect of hypoxia on ets-1 protein expression densitometry measurement.

6.3.2 Effect of hypoxia on ets-1 and HIF-1 α protein expression in rat tissue explants

Results for ets-1 protein expression from the rat tissue explants were not analysable by Sion software. An example of these westerns can be seen in Figure 18. Westerns for HIF-1 α protein expression showed analysable results as seen figures 19 to 22, however no discernable pattern for levels of HIF-1 α expression between hypoxic and normoxic, adjuvant and naïve samples could be detected.



Key

NN1 = Normoxic Naïve Sample Number 1

NN2 = Normoxic Naïve Sample Number 2

HN1 = Hypoxic Naïve Sample Number 1

HN2 = Hypoxic Naïve Sample Number 2

NA1 = Normoxic Adjuvant Sample Number 1

NA2 = Normoxic Adjuvant Sample Number 2

HA1 = Hypoxic Adjuvant Sample Number 1

HA2 = Hypoxic Adjuvant Sample Number 2

+ve Control , -ve control

Figure 18. Effect of hypoxia on ets-1 protein expression by adjuvant and naïve rat tissue explants.

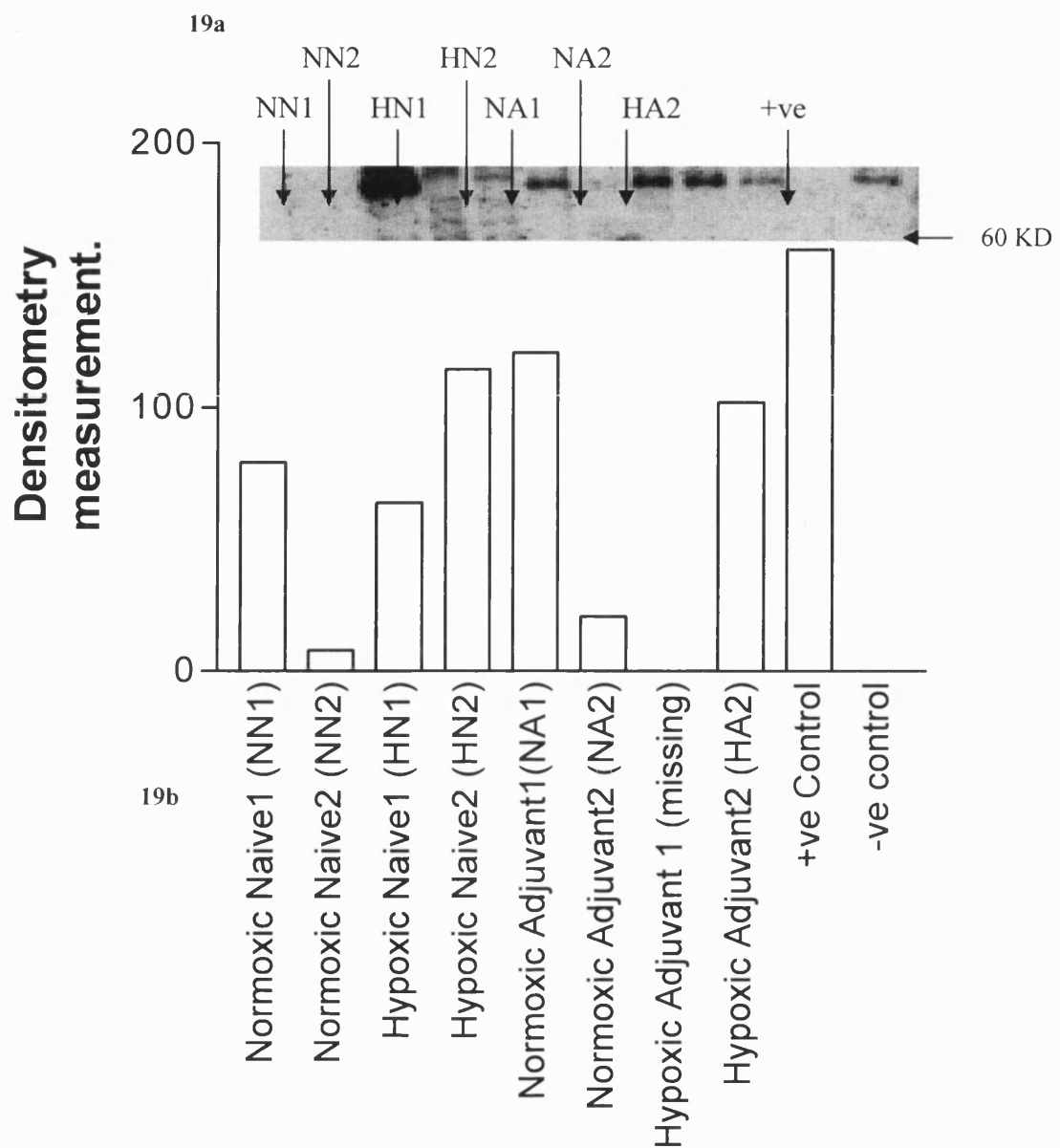


Figure 19a. Western Blot: Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants.

Figure 19b. Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants densitometry measurement.

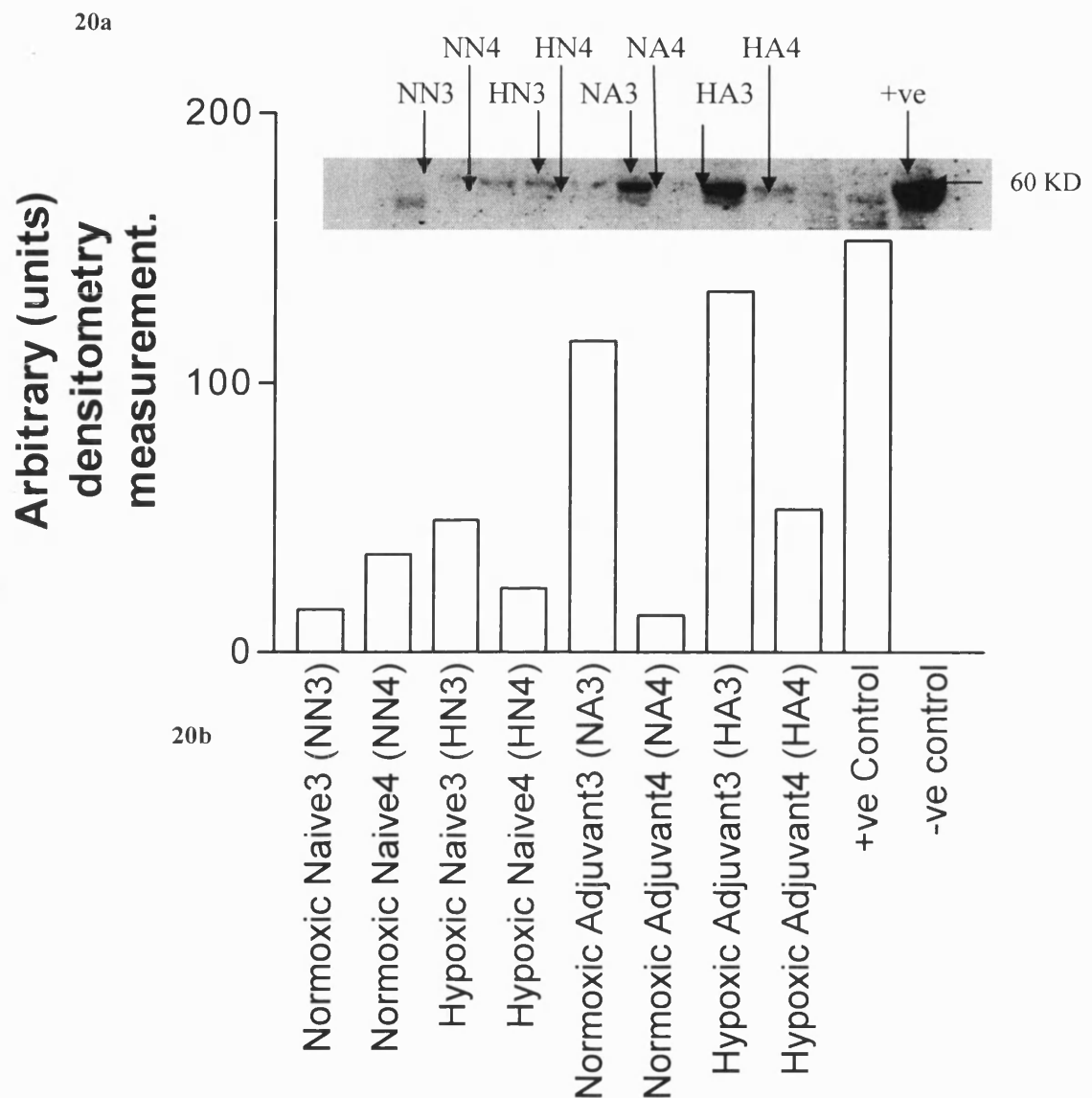


Figure 20a. Western Blot: Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants.

Figure 20b. Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants densitometry measurement.

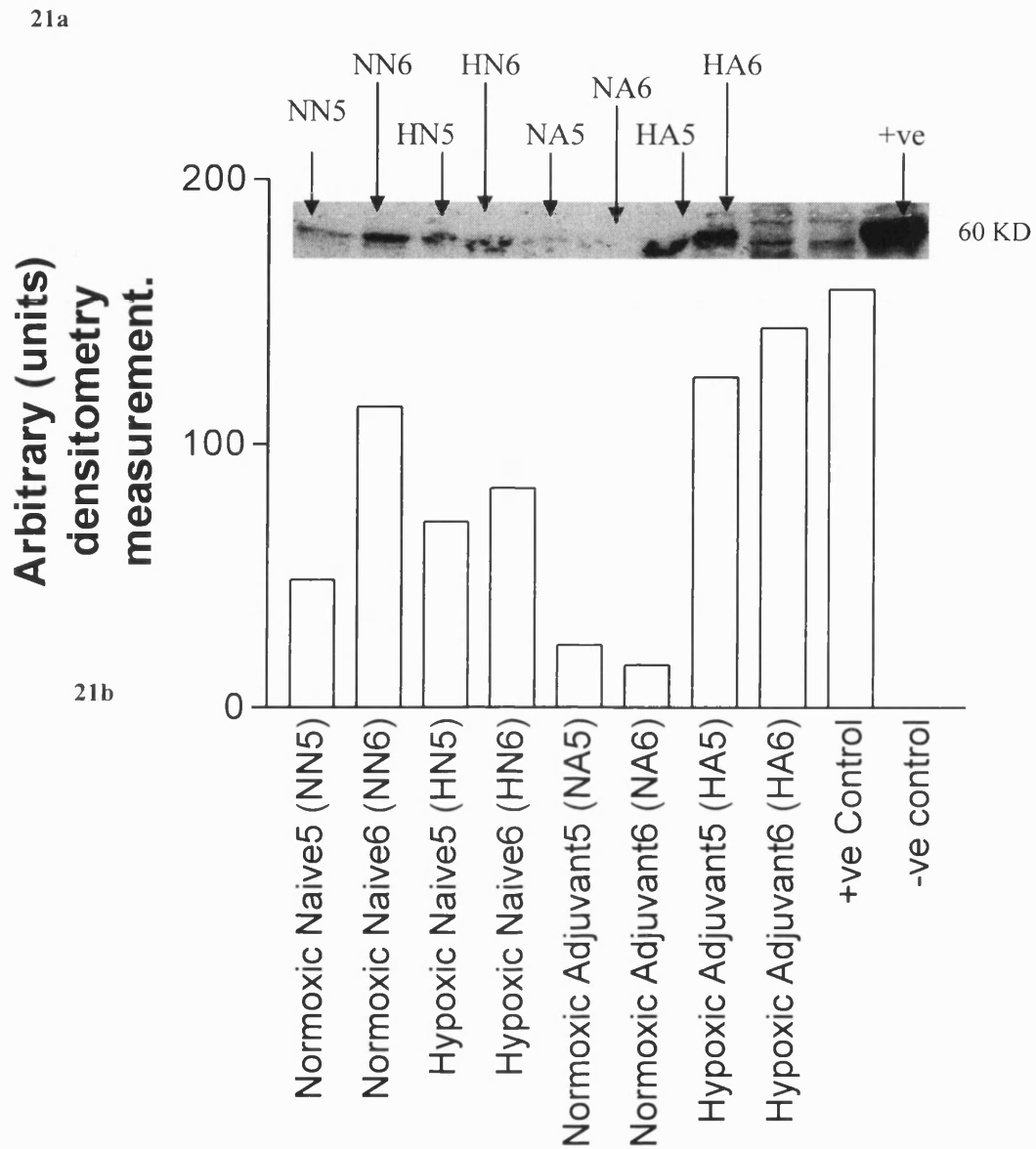


Figure 21a. Western Blot: Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants.

Figure 21b. Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants densitometry measurement.

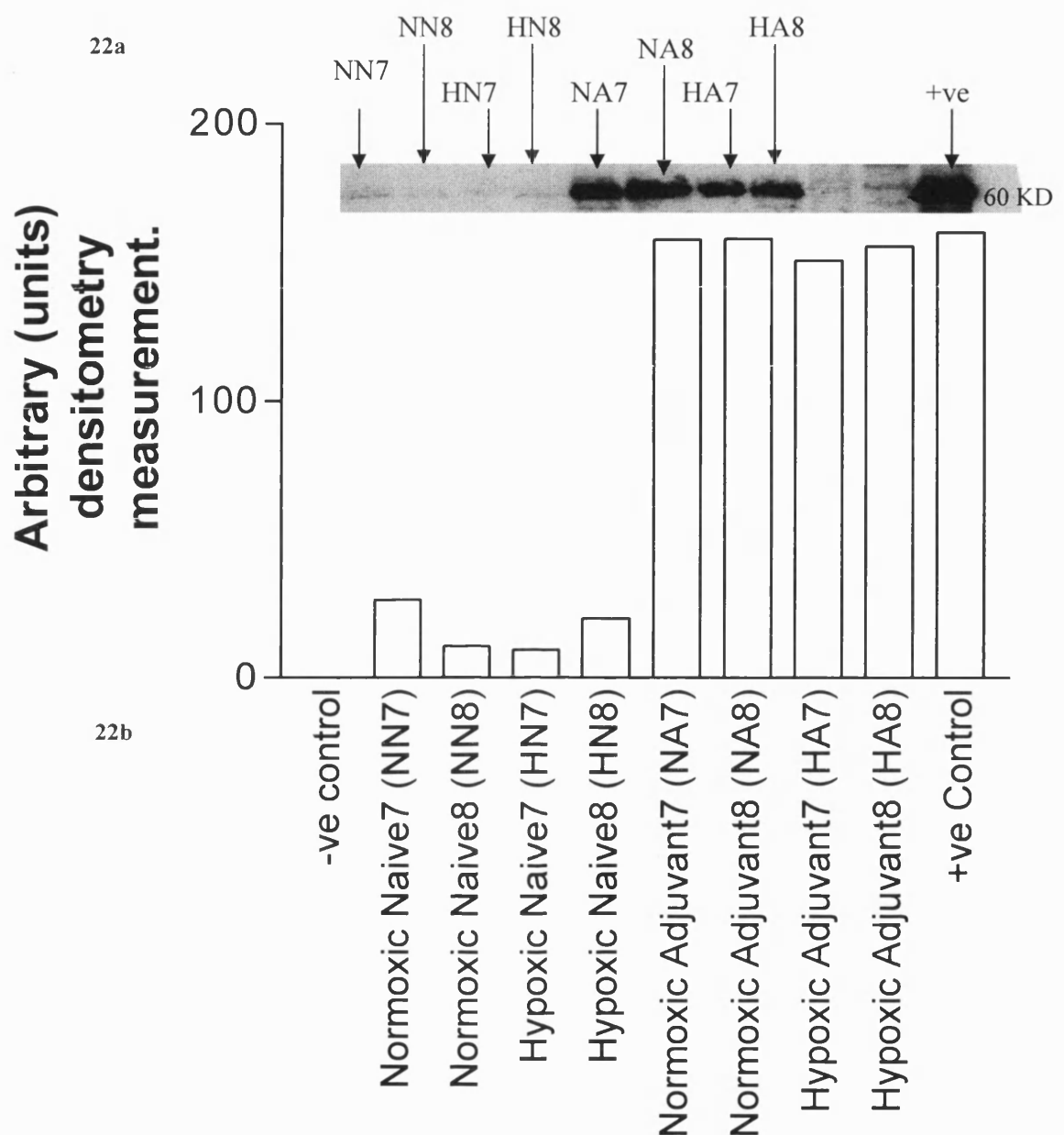


Figure 22a. Western Blot: Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants.

Figure 22b. Effect of hypoxia on HIF-1 α protein expression by adjuvant and naïve rat tissue explants densitometry measurement.

6.3.3 Histological assessment of rat tissue explants from normoxic and hypoxic culture

A representative set of stained sections presented in Plates. 6.1 to 6.6 show the histology of patella suspensory ligaments that have been cultured at either 0% or 20% oxygen compared to patella suspensory ligaments that have just been fixed and embedded without culture. These slides show a qualitative reduction in the number of cells present within the tissue of cultured samples compared to those that have not been cultured. This result was observable in both naïve and inflamed adjuvant samples.

Plate 6.1 Adjuvant knee synovium (x5, wax section, haematoxylin and eosin) with a high clinical score (3). Not cultured, observe high density of cells proliferating at the synovium edge [arrows] compared to plate 7.2.

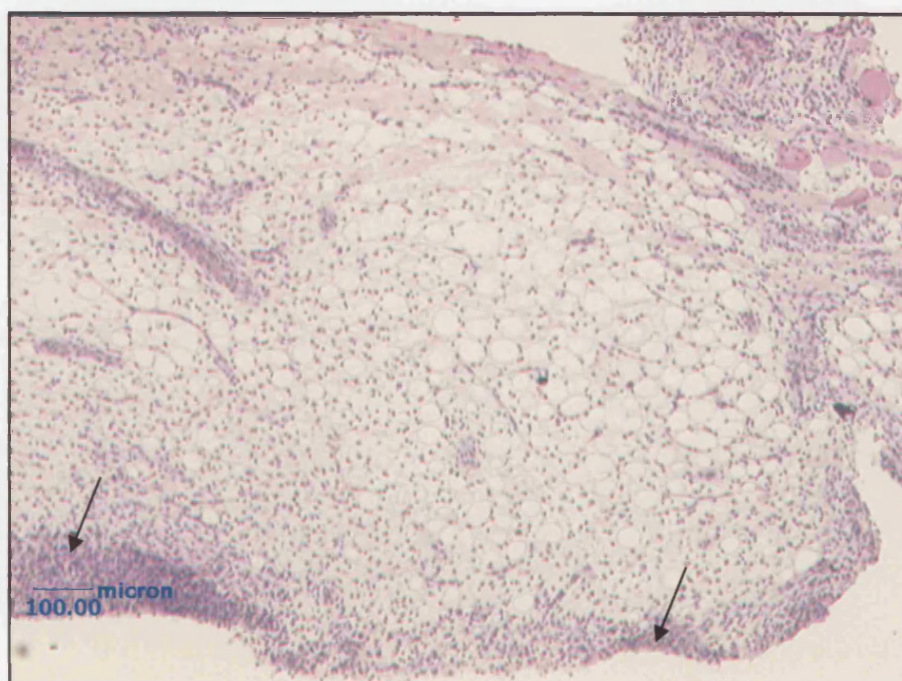


Plate 6.2 Adjuvant knee synovium (x5, wax section, haematoxylin and eosin) with a high clinical score (3). Cultured in 0% O₂, observe lack of cells throughout the synovium [S].

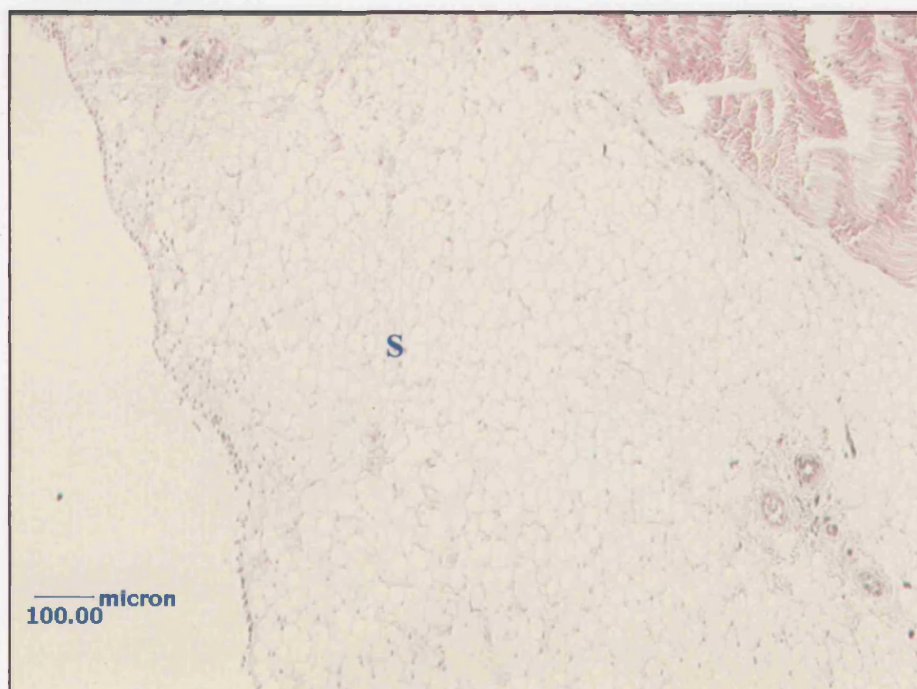


Plate 6.3 Adjuvant knee synovium (x5, wax section, haematoxylin and eosin) with a high clinical score (3). Cultured in 20%O₂, there are few cells scattered in the synovium and a greater concentration at the synovium edge [arrows].

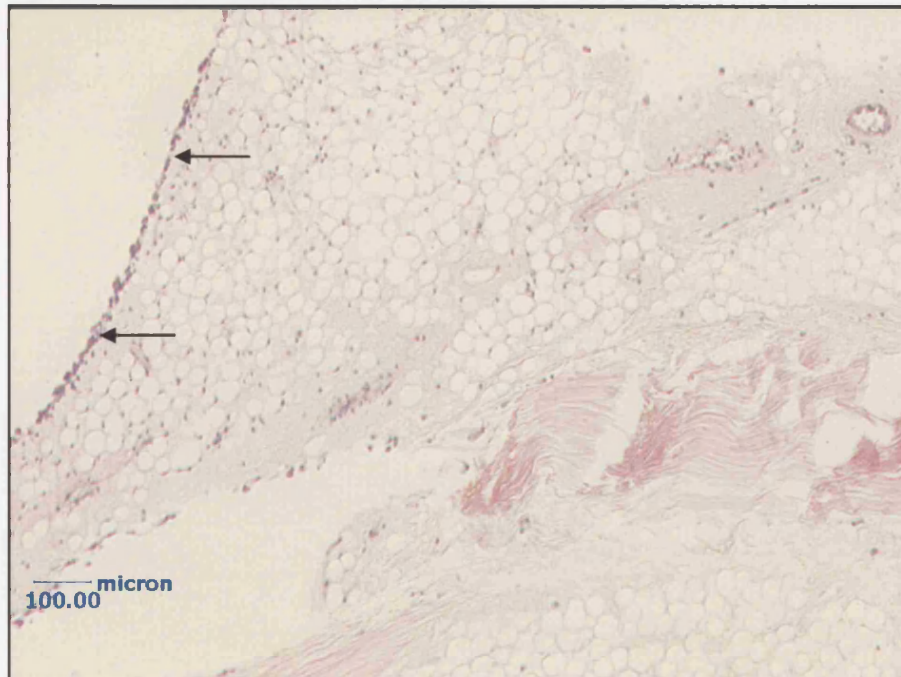


Plate 6.4 Naive knee synovium (x5, wax section, haematoxylin and eosin) with zero clinical score. Not cultured, observe greater cellularity throughout the synovium [S] compared to plate 7.5.

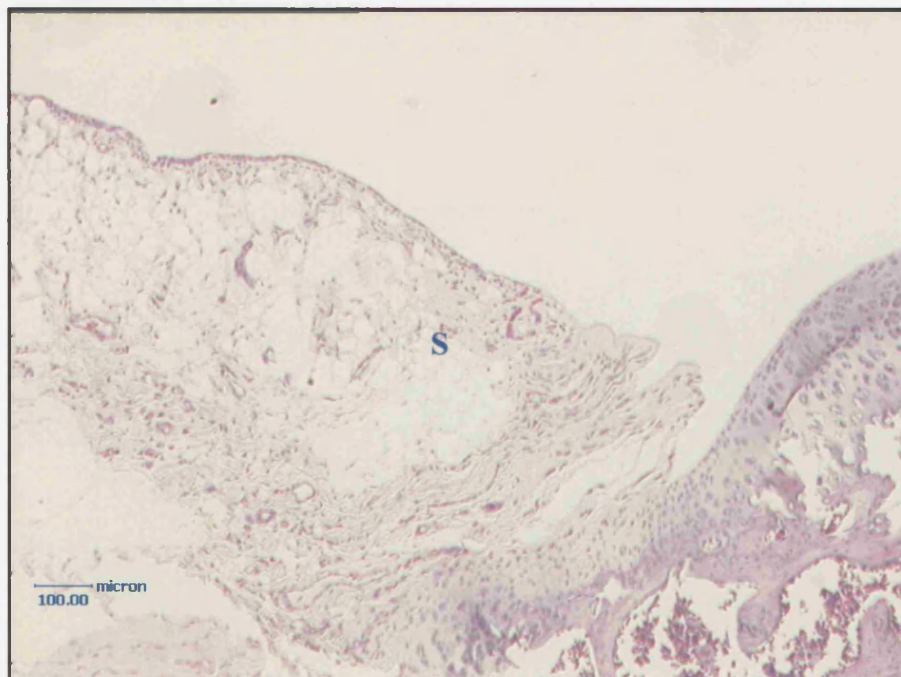


Plate 6.5 Naive knee synovium (x5, wax section, haematoxylin and eosin) with a zero clinical score. Cultured in 0% O₂. There are few cells in the synovium [S].

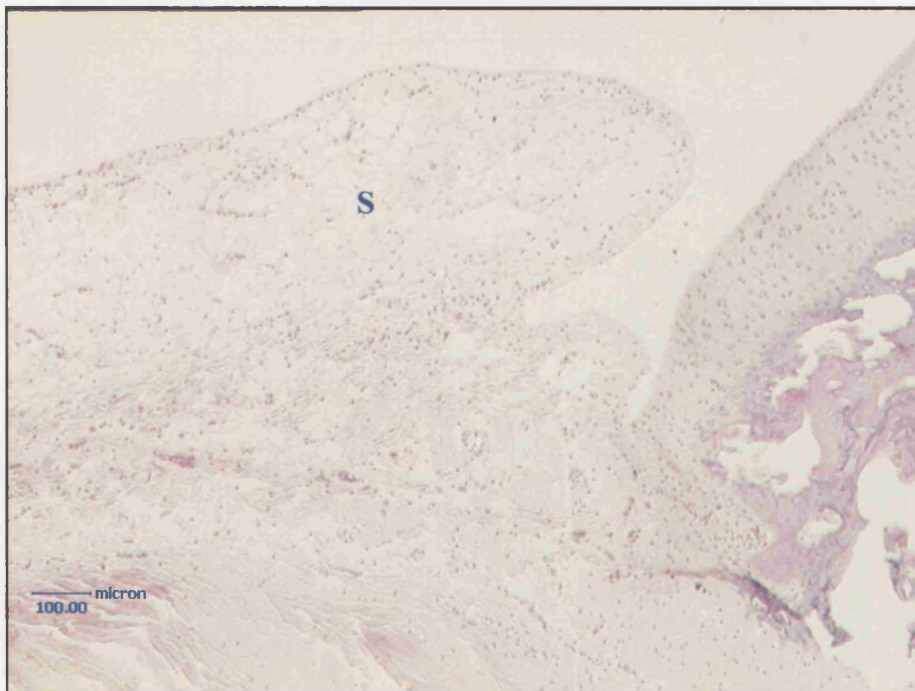


Plate 6.6 Naive knee synovium (x5, wax section, haematoxylin and eosin) with a zero clinical score. Cultured in 20% O₂. There are few cells in the synovium [S] and small aggregations at the synovium edge [arrows].



Plate 6.1 is a haematoxylin and eosin stained section of a patella suspensory ligament from an adjuvant rat with a high clinical score that has not been cultured. Observe the high concentration of cells in the synovium edge compared to plate 6.2 where an explant with identical clinical score has been cultured for 14 days in 0% O₂, and is essentially acellular. Under higher oxygen concentrations it would be expected that perhaps there would be more cells present but again Plate 6.3 shows a haematoxylin and eosin stained section of a patella suspensory ligament from the same adjuvant rat as plate 6.2 that has been cultured for 14 days in 20% O₂, with few cells in the synovium. The difference here is that small aggregations of cells appear to be using the synovium edge as a growth matrix.

A similar situation is observable in the naïve samples. Plate 6.4 for example is a haematoxylin and eosin stained section of a patella suspensory ligament from a naïve rat with a zero clinical score that has not been cultured. There is a high concentration of cells at the synovium edge compared to plate 6.5 with a matched zero clinical score that has been cultured for 14 days in 0% O₂. There are few cells in the synovium. As in the adjuvant rats, higher oxygen concentrations are not associated with greater cellularity as shown in Plate 6.6. This plate shows a haematoxylin and eosin stained section of a patella suspensory ligament from the same naïve rat as plate 6.5 that has been cultured for 14 days in 20% O₂, with a low concentration of cells in the synovium.

6.4 Discussion

These studies represent preliminary results. Of studies that warrant further investigation.

The antibody to HIF-1 α detects a band at 110 KD, however in all of my western blots the only band visible was at 60 KD. Having run appropriate controls (both negative and positive) and discussing the results with the antibody manufacturer, I was left to conclude that as all my negative controls had no bands and all my positive controls had a band at 60 KD that this band at was HIF-1 α which had probably been broken down during protein retrieval. The band is possibly a dimer; hence the occasional double band appearance seen in figure 16a. Results from the rheumatoid knee synovial fibroblast western blots clearly show an increase in HIF-1 α protein expression and a concurrent increase in ets1 protein expression when cultured in 0% oxygen compared to cells cultured in 20% oxygen. This is the first work in this thesis involving human cells instead of rat tissue and it reassuring to see that results corroborate my studies linking an increase in hypoxia with increase in HIF-1 α and ets1 expression in the adjuvant arthritis model. Also because these cells are synovial fibroblasts it substantiates my previously mentioned theory that an increase in ets-1 expression does not have to be associated with angiogenesis in the rheumatoid synovium but could be more involved with the invasive characteristics of RA synovium. This is not implausible as others have demonstrated that ets-1 transcription factor up-regulation correlates with tumour invasion (Vandebunder et al 1994, Wernert et al., 1994). If time had allowed it would have been interesting to see if hypoxic culture conditions also lead upregulation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) like collagenase 1 (MMP-1), stromelysin (MMP-

3), gelatinase B (MMP-9) and TIMP-1. All are gene targets of ets-1 and have been shown to be elevated in patients with RA (Vincenti et al., 1994, Lelievre et al., 2001 and Klimiuk et al., 2002).

From this work and my previous studies in adjuvant arthritis it would have been logical to hypothesise that rat synovium either inflamed or naive would show an increased expression of HIF-1 α and ets1 protein under conditions of hypoxia. However, it can be seen from my results in this section (6.3.2) that this was not the case *ex vivo*. The results are inconsistent and there seems to be no pattern to the transcription factor protein expression. Repetition of these westerns did not result in any improvement of outcomes.

I hypothesised that there was a reduction in cells growing out from the explants which lead to the protein being retrieved from non viable cells and cellular debris. . Therefore explants cultured were removed for subsequent histological examination and these did show a remarkable level of acellularity. An additional problem caused by a large amount of cell death is this may also result in antigen breakdown and inconsistent results. I suggest that the lack of cellular proliferation may be due to a deficiency of cytokines in this experimental system. It would be interesting to observe the effects of the addition of inflammatory cytokines, for example basic fibroblastic growth factor which has been shown to simulate growth in many cells (Goddard et al., 1992) and transforming growth factor β (TGF- β), because it modulates fibroblast recruitment and proliferation (Berse, 1999).

I feel that some of the most potentially interesting results of this chapter come from embedded rat patella suspensory ligaments that had been cultured, sectioned and compared to tissue that had not been cultured, section 6.3.3. The histology of sections shows a

qualitative reduction in the number of cells present within the tissue of cultured samples compared to those that have not been cultured irrespective of oxygen concentration. This result was observable in both naïve and inflamed adjuvant samples. There are a number of possible explanations for this the most probable being that whilst undergoing 14 days of culture, cell death occurred and this would account for the lack of cells in the tissue explants and also the poor results observed in the western blots mentioned earlier. But could there be more to this than cell death? Observation of the slides showed no cell or nuclear debris that I would expect to see if there was apoptosis occurring. In rheumatoid synovium it has been suggested that part of the proliferative characteristic of RA synovium may be due to impaired apoptosis (Nakajima et al, 1995). Franz et al (2000) found marked expression of the antiapoptotic molecule sentrin in rheumatoid synovial tissue, but not in osteoarthritis or normal synovium. This work has been supported by animal models of synovium which also demonstrate reduced apoptosis (Soo, 1999). Time and resources allowing, it would have been interesting to have measured markers of apoptosis in my study.

If the cells are not dying where are they? A theory I would like to propose is that the cells may have migrated, in search of more favorable oxygen concentrations. After all 0% oxygen is too little oxygen to sustain cells that favor aerobic conditions and 20% oxygen is probably hyperoxia compared to the normal oxygen tensions of the knee. This might account for why the cells still appear to be growing along the edges of rat tissue explants cultured in 20% oxygen. There is no evidence in my current study to substantiate this theory but perhaps it warrants further investigation through the study of markers of motility in inflamed synoviocytes. Could this be another characteristic of the transformed

synoviocyte, the ability to migrate to environments of optimal oxygen concentrations and therefore away from areas of hypoxia?

CHAPTER 7: Summary of Conclusions and General Discussion

The hypothesis behind this thesis is that global hypoxia in the diseased joint includes hypoxic synovial cells that produce pro-inflammatory cytokines and transcription factors that provide a potential target for bio-reductive and other novel therapies. The aim of my study was to use the adjuvant arthritis model to identify at what stage during disease progression the joint becomes hypoxic. To examine the phenotype of hypoxic cells and to identify cytokines and transcription factors produced.

The adjuvant arthritis model was used in this study because historically it has been used in previous studies demonstrating hypoxia in the inflamed joint (Bruce, 1992), the resources were most accessible and it presents the disease aspects of interest namely: inflammation, synovial hyperplasia, pannus formation and erosion of bone and cartilage. However, unlike the Bruce (1992) study which measured oxygen tensions in the inflamed joint with a probe, my study aimed to identify hypoxia in the inflamed joint at a cellular level.

To achieve this hypoxyprobe-1, a commercially available injectable preparation of pimonidazole (a modified 2-nitromidazole) was employed. This is taken up by all tissues in the body but binds only to cells with a pO_2 of 10 mm Hg (or less) at 37°C. Hypoxyprobe-1MAB1 is a monoclonal antibody that binds to specific protein adducts of Hypoxyprobe produced in hypoxic cells and this allows their detection in tissues *ex vivo*. At the time that this study was carried out pimonidazole had only been used to measure low oxygen in animal and human tumours as an index of radiation resistance (Kim et al., 1993, Kennedy et al., 1997). As this study used the model adjuvant arthritis in the Lewis rat a pilot study (chapter 3) was carried out to determine an appropriate method and preliminary qualitative

results for hypoxic cell labeling in the adjuvant rat. From this study, a 6-hour time point for dosage of the animals prior to termination was chosen because it was the shortest time that gave specific labeling.

The objective of chapter 4 was to quantitatively compare the level of hypoxia in the adjuvant arthritis inflamed joint to the naive matched controls and establish at what point during the disease progression the joint becomes hypoxic. This study showed, for the first time that as the clinical symptoms of adjuvant arthritis develop so the inflamed joint becomes hypoxic and that this hypoxia is significantly higher than in naïve controls. Hypoxyprobe-1 adducts in the adjuvant foot tissue involved all structures including the synovium, pannus, bone marrow and articular cartilage chondrocytes. Further work (chapter 5) was carried out to try and identify the cell types that labelled with hypoxyprobe-1 adduct. Several key markers were examined: fibroblast and macrophage markers; cytokines, iNOS, VCAM-1 and VEGF; transcription factors, HIF-1 α and ets-1. It is unfortunate that the studies in this chapter failed to identify specific cell type markers associated with hypoxic cells. This is probably due to problems I had maintaining sample preservation and antigenicity through the process of fixation and decalcification required for the examination of bony samples. Indeed, an increase in the cells expressing ED2 and to lesser extent ED1 macrophage markers and an increase in both iNOS and VEGF were observed, though there was no co-localization with areas of hypoxia. From this I conclude that hypoxia does not play as significant a role in their expression as do other factors. These are likely to be cytokines like TNF α and IL-1. Such results support recently published work that indicates that an increase of the VEGF/KDR (kinase insert domain

protein receptor) pathway can occur independent of increases in HIF-alpha expression in rheumatoid arthritis (Giatromanolaki et al., 2003).

Like hypoxyprobe, hypoxia inducible factor-1 α an endogenous marker of hypoxia involved all structures including the synovium, pannus, bone marrow and articular cartilage chondrocytes of the adjuvant foot tissue however this contrasts with work carried out by Hollander et al., (2001), who demonstrated that HIF-1 α localised to macrophages. My study compares very well to similar studies carried out more recently in rheumatoid synovium. Two groups Hitchon et al., (2002) and Giatromanolaki et al., (2003) published immunohistochemical studies that localized HIF-1 α in synoviocytes lining the inflamed synovial tissue. Finally in this chapter I demonstrated an increased cellular hypoxia to be associated with a concurrent increase in the expression of ets-1 transcription factor protein during the inflammatory response in adjuvant rat joints. From this I concluded that hypoxia and HIF-1 α may be involved in the upregulation of ets-1 during joint inflammation.

My final experimental chapter (chapter 6) sought to determine ets-1 and HIF-1 expression *in vitro*, in hypoxic rheumatoid synovial fibroblasts (RSF) and hypoxic adjuvant rat synovium. The RA fibroblasts showed an increase in HIF-1 α protein expression and a concurrent increase in ets1 protein expression when cultured in 0% oxygen conditions compared to cells cultured in 20% oxygen conditions. This work corroborates my theory that an increase in ets-1 expression does not have to be associated with angiogenesis in the rheumatoid synovium but could be more involved with the invasive characteristics of RA synovium. Others have demonstrated that ets-1 transcription factor up-regulation correlates with tumour invasion (Vandenbunder et al 1994, Wernert et al., 1994). My localisation of ets-1 in the invasive pannus is indicative of ets-1 involvement in the invasion of synovial

joint cartilage and bone by proliferating synovium. This theory is further supported by the broad range of target genes for *ets-1*: matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) such as collagenase 1 (MMP-1), stromelysin (MMP-3), gelatinase B (MMP-9) and TIMP-1 are all gene targets of *ets-1* (Lelievre et al., 2001) and have been shown to be elevated in patients with RA (Vincenti et al., 1994 and Klimiuk et al., 2002). Indeed Czuwara-Ladykowska (2002) has shown that elevated *ets1* expression in fibroblasts alters their response to TGF β in favour of matrix degradation. From the evidence of my studies I propose that hypoxia arises in the inflamed rheumatoid joint as a result of inflamed and proliferating tissue has a high metabolic demand but poor neovasculature, which is unable to meet the oxygen demand. This leads to increased HIF-1 α expression; HIF-1 α binds to hypoxic response like binding sequences on the *ets-1* promoter which leads to increased expression of *ets1* by fibroblast like synoviocytes. This throws a switch in the direction of matrix degradation and thus the destruction of the joint.

Increased hypoxia has been associated with a number of diseases such as cancer (Brahimi-Horn et al., 2001; and Giatromanolaki and Harris, 2001); cardiovascular disease (Schmedtje and Ji, 1998), respiratory disease (Semenza, 2000 and Semenza, 2001) and rheumatoid arthritis (Mapp et al., 1995 and Bodamyali et al., 1998) and this has lead to the concept of hypoxia-targeted therapies. Some of the first therapies to target hypoxic tissue in disease were bioreductive drugs. Bioreductive drugs are prodrugs that are metabolised to either generate or release an active species in the presence of reducing environment (e.g., hypoxia or reductive enzymes). These reducing enzymes include NADP(H) oxidoreductase, NADPH cytochrome c (P450) reductase, cytochrome b5 reductase, carbonyl reductase and xanthine oxidase/xanthine dehydrogenase (Jaffar et al., 2001). In

particular the xanthine oxidase system has been demonstrated in the rheumatoid synovium (Stevens et al., 1991).

Gene therapy is the introduction of therapeutic genes to human cells for the treatment of disease (Hollander et al., 2001). Hypoxia inducible factor 1 α expression is abundant in rheumatoid synovium (Hitchon et al., 2002, Giatromanolaki et al., 2003) so strategies that target HIF-1 could be effective in treating rheumatoid arthritis. In cancer this goal has already been achieved through the use of HIF-1 anti-sense (Sun et al., 2001) and through inhibiting the interaction between HIF-1 and the specific co-factors required for HIF-1 mediated transcription (Kung et al., 2000).

Hypoxia can also be used to target gene therapy to certain diseased areas. This concept exploits the fact that HIF-1 is degraded by exposure to oxygen but activated and stabilized under conditions of hypoxia (Wiesener et al., 1998). The expression of a therapeutic gene of interest is regulated by the inclusion of a hypoxia responsive element (HRE) upstream of the therapeutic gene in an expression vector. This concept was first demonstrated using human fibrosarcoma cells (HT1080) where the expression of a stably transfected gene could be induced by hypoxia (both in vitro and in vivo) by the presence of a HRE enhancer sequence (Dachs et al., 1997). It has been shown that macrophages can be transfected with HRE-regulated transgenes and used to deliver them to hypoxic sites in tumours (Griffiths et al., 2000).

At this time it is not possible to know if ets-1 has a role in hypoxia-regulated gene therapy for arthritis, as it is not fully understood whether other factors present in-vivo would also activate ets-1 in a gene transfer delivery vehicle. Nevertheless, because this study has

demonstrated both increased hypoxia and an increase in ets-1 expression at the site of cartilage and bone destruction, it is likely that HIF-1 mediated systems could be used.

This study clearly shows adjuvant arthritis to be a suitable model for *in vivo* research into the likely efficacy of hypoxia-regulated therapies in the treatment of rheumatoid arthritis.

CHAPTER 8: Future Work

This thesis demonstrated the labeling hypoxic cells in adjuvant arthritis a model that mirrors much of the pathology of rheumatoid arthritis (RA). It showed, for the first time, that as the clinical symptoms of adjuvant arthritis develops so the inflamed joint becomes hypoxic and that this hypoxia is significantly higher than naïve controls. The main aim of future studies would be to see if these results could be replicated in patients. Although this study would require ethical consideration it is within the realms of possibility, as Hypoxyprobe-1 is already approved for use in human cancer research.

It is unfortunate that my studies have not identified specific cell type marker associated with cells labelling with hypoxyprobe. This was probably due to antigenicity loss during sample processing. It would be useful for future studies to investigate methods for achieving better antigen preservation during processing of boney tissues. However, in the end even with the best antibody specificity possible, it is probable that the hypoxic cells identified in the inflamed joint are a heterogeneous population with phenotypical characteristics from both fibroblast and macrophage lineage. Further work should be carried out to improve the western blot methodology and maybe even look into messenger RNA for upregulation of matrix metalloproteinases (MMPs) in the *in vitro* system employed to investigate the affect of induced hypoxia on normal and inflamed synovium. It would also be interesting to observe the effects of the addition of inflammatory cytokines to the *in vitro* studies for example basic fibroblastic growth factor which has been shown to simulate growth in many cells (Goddard et al., 1992) and transforming growth factor β (TGF- β), because it modulates fibroblast recruitment and proliferation (Berse, 1999).

It would also be desirable to investigate further the upstream control of ets-1 expression in the inflamed synovium,

I feel some of the most curious results from my study came from a decision to embed some of the rat patella suspensory ligaments and compare the histology of sections that had been cultured at either 0% or 20% oxygen compared to patella suspensory ligaments that have just been fixed and embedded without culture. These slides show a qualitative reduction in the number of cells present within the tissue of cultured samples compared to those that have not been cultured. I feel this is worth further investigation, since in rheumatoid synovium it has been suggested that part of the proliferative characteristic of RA synovium may be due to impaired apoptosis (Nakajima et al, 1995). It would be interesting to measure markers of apoptosis in a repetition of my study. A theory I have proposed is that the cells may have migrated, in search of more favorable oxygen concentrations. There is no evidence in my current study to substantiate this theory but perhaps it could be further investigated through the study of markers of motility in inflamed synoviocytes.

Finally I believe my study has shown adjuvant arthritis to be a suitable model for future *in vivo* research into the likely efficacy of hypoxia-regulated therapies like bioreductive and hypoxia mediated gene therapy in the treatment of rheumatoid arthritis.

References

Abramson, S., Weissmann G. The mechanisms of action of nonsteroidal antiinflammatory drugs. *Arthritis and Rheumatism* **32**, 1-9 (1989).

Abramson, S., Amin A. Blocking the effects of IL-1 in rheumatoid arthritis protects bone and cartilage. *Rheumatology (Oxford)*. **41**, 972-80 (2002).

Adam, M., Dorie MJ, Brown JM. Oxygen tension measurements of tumors growing in mice. *Int J Radiat Oncol Biol Phys* **45**, 171-80 (1999).

Adams, G., Stratford IJ. Bioreductive drugs for cancer therapy: the search for tumor specificity. *Int J Radiat Oncol Biol Phys*. **29**, 231-8 (1994).

Airley, R., Loncaster, J, Davidson S, Bromley M, Roberts S, Patterson A, Hunter R, Stratford I, West C. Glucose transporter glut-1 expression correlates with tumor hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix. *Clin Cancer Res*. **7**, 928-34 (2001).

Albina, J., Mastrofrancesco, B, Vessella JA, Louis CA, Henry WL Jr, Reichner JS. HIF-1 expression in healing wounds: HIF-1alpha induction in primary inflammatory cells by TNF-alpha. *Am J Physiol Cell Physiol*. **281**, C1971-7 (2001).

Allen, R., Outhwaite JM, Morris CJ, Blake DR. Xanthine oxidoreductase is present in human synovium. *Ann Rheum Dis*. **46**, 843-5 (1987).

Allen, R., Tresini M. Oxidative stress and gene regulation. *Free Radic Biol Med* **28**, 463-99 (2000).

Amin, A. Type II interleukin-1beta receptor: a candidate for gene therapy in human arthritis. *Clin Orthop*. **379 Suppl**, S179-88. (2000).

Andrews, F., Morris, CJ, Lewis EJ and Blake DR. Effect of nutritional iron deficiency on acute and chronic inflammation. *Annals of the Rheumatic Diseases*. **46**, 859-865 (1987a).

Andrews, F., Morris, CJ, Kondratowicz G and Blake DR. Effect of iron chelation on inflammatory joint disease. *Annals of the Rheumatic Diseases*. **46**, 327-333 (1987b).

Angel, P., Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* **1072**, 129-57 (1991).

Arend, W., Malyak M, Guthridge CJ, Gabay C. Interleukin-1 receptor antagonist: role in biology. *Annu Rev Immunol*. **16**, 27-55 (1998).

Armstrong, L., Bornstein P. Thrombospondins 1 and 2 function as inhibitors of angiogenesis. *Matrix Biol* **22**, 63-71 (2003).

Arteel, G., Thurman, RG, Raleigh JA. Reductive metabolism of the hypoxia marker pimonidazole is regulated by oxygen tension independent of the pyridine nucleotide redox state. *Eur J Biochem* **253**, 743-50 (1998).

Aupperle, K., Boyle, DL, Hendrix M, Seftor EA, Zvalier NJ, Barbosa M and Firestein GS. Regulation of synoviocyte proliferation, apoptosis, and invasion by the p53 tumour suppressor gene. *American Journal of Pathology* **152**, 1091-98 (1998).

Azuma, C., Raleigh, JA, Thrall DE. Longevity of pimonidazole adducts in spontaneous canine tumors as an estimate of hypoxic cell lifetime. *Radiat Res* **148**, 35-42 (1997).

Babior, B. Oxygen-dependent microbial killing by phagocytes. *The New England Journal of Medicine* **298**, 659-668 (1978).

Baeuerle, P., Baichwal VR. NF-kappa B as a frequent target for immunosuppressive and anti-inflammatory molecules. *Adv.Immunol* **65**, 111-37 (1997).

Baier, A., Meineckel I, Gay S, Pap T. Apoptosis in rheumatoid arthritis. *Curr Opin Rheumatol* **15**, 274-9 (2003).

Balandraud, N., Meynard JB, Auger I, Sovran H, Mugnier B, Revirion D, Roudier J, Roudier C. Epstein-Barr virus load in the peripheral blood of patients with rheumatoid arthritis: accurate quantification using real-time polymerase chain reaction. *Arthritis and Rheumatism* **48**, 1223-8 (2003).

BARLAND, P., NOVIKOFF, AB, HAMERMAN D. Electron microscopy of the human synovial membrane. *J Cell Biol.* **14**, 207-20 (1962).

Bashir, S., Harris G, Denman MA, Blake DR, Winyard PG. Oxidative DNA damage and cellular sensitivity to oxidative stress in human autoimmune diseases. *Ann Rheum Dis* **52**, 659-66 (1993).

Battisto, J., Smith RN, Beckman K, Sternlicht M, Welles WL. Susceptibility to adjuvant arthritis in DA and F344 rats. A dominant trait controlled by an autosomal gene locus linked to the major histocompatibility complex. *Arthritis Rheum.* **25**, 1194-200 (1982).

Bauerova, K., Bezek, A. Role of reactive oxygen and nitrogen species in etiopathogenesis of rheumatoid arthritis. *Gen Physiol Biophys.* **18**, 15-20. (1999).

Bentin, J. Mechanism of action of cyclosporin in rheumatoid arthritis. *Clin Rheumatol.* **14**, 22-5 (1995).

Bersani-Amado, C., Duarte AJ, Tanji MM, Cianga M, Jancar S. Comparative study of adjuvant induced arthritis in susceptible and resistant strains of rats. III. Analysis of lymphocyte subpopulations. *J Rheumatol* **17**, 153-8 (1990).

Berse, B., Hunt, JA, Diegel RJ, Morganelli P, Yeo K, Brown F, Fava RA.(). Hypoxia augments cytokine (transforming growth factor-beta (TGF-beta) and IL-1)-induced vascular endothelial growth factor secretion by human synovial fibroblasts. *Clin Exp Immunol.* **115**, 176-82 (1999).

Billingham, M., Carney, S, Butler R and Colston MJ. A mycobacterial 65-kD heat shock protein induces antigen-specific suppression of adjuvant arthritis, but is not itself arthritogenic. *Journal of Experimental Medicine.* **171**, 339-344 (1990).

Billingham, M. in *Mechanisms and Models in Rheumatoid Arthritis* (ed. Henderson, B., Edwards JCW, Pettipher ER) (Academic Press, 1995).

Blake, D., Winyard, P, Scott DG, Brailsford S, Blann A, Lunec J. Endothelial cell cytotoxicity in inflammatory vascular diseases--the possible role of oxidised lipoproteins. *Ann Rheum Dis.* **44**, 176-82. (1985).

Blake, D., Unsworth, J, Outhwaite M, Morris CJ, Merry P, Kidd BL, Ballard R, Gray L, Lunec J. Hypoxic-reperfusion injury in the inflamed human joint. *The Lancet* **1**, 289-93 (1989).

Blake, D. Measuring outcome in rheumatoid arthritis--which measures are suitable for routine clinical use? *Br J Rheumatol.* **35**, 301 (1996).

Blake, D., Stevens, CR, Sahinoglu T, Ellis G, Gaffney K, Edmonds S, Benboubetra M, Harrison R, Jawed S, Kanczler J, Millar TM, Winyard PG and

Zhang Z . Xanthine oxidase: four roles for the enzyme in rheumatoid pathology. *Biochemical Society Transactions*. **25**, 812-816 (1997).

Bodamyali, T., Stevens CR, Billingham MEJ, Blake DR. Influence of hypoxia in inflammatory synovitis. *Ann Rheum Dis* **57**, 703-10 (1998).

Borderie, D., Hilliquin P, Hernvann A, Lemarechal H, Menkes CJ, Ekindjian OG. Apoptosis induced by nitric oxide is associated with nuclear p53 protein expression in cultured osteoarthritic synoviocytes. *Osteoarthritis Cartilage* **7**, 203-13 (1999).

Brahimi-Horn, C., Berra E, Pouyssegur J. Hypoxia: the tumor's gateway to progression along the angiogenic pathway. *Trends Cell Biol* **11**, S32-6 (2001).

Brenchley, P. Angiogenesis in inflammatory joint disease: a target for therapeutic intervention. *Clin Exp Immunol*. **121**, 426-9 (2000).

Bresnihan, B., Alvaro-Gracia JM, Cobby M, Doherty M, Domljan Z, Emery P, Nuki G, Pavelka K, Rau R, Rozman B, Watt I, Williams B, Aitchison R, McCabe D, Musikic P. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis and Rheumatism* **41**, 2196-204 (1998).

Bruce, A. in *Faculty of Science* (University of London, 1992).

Bucala, R., Ritchlin C, Winchester R, Cerami A. Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. *J Exp Med* **173**, 569-74 (1991).

Bulter, D., Leizer, T, Hamilton JA. Stimulation of human synovial fibroblast DNA synthesis by platelet-derived growth factor and fibroblast growth factor. Differences to the activation by IL-1. *Journal of Immunology*. **142**, 3098-103 (1989).

Burdon, R. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radical Biology and Medicine*. **18**, 775-94 (1995).

Burdon, R. Control of cell proliferation by reactive oxygen species. *Biochem Soc Trans* **24**, 1028-32 (1996).

Burmester, G., Daser, A, Kamradt T, Krause A, Mitchison NA, Sieper J and Wolf N. Immunology of reactive arthritides. *Annual Review of Immunology*. **13**, 229-50 (1995).

Butler, D., Leizer T, Hamilton JA. Stimulation of human synovial fibroblast DNA synthesis by platelet-derived growth factor and fibroblast growth factor. Differences to the activation by IL-1. *J Immunol* **142**, 3098-103 (1989).

Buttgereit, F., Brink I, Thiele B, Burmester GR, Hiepe F, Hall ED. Effects of methylprednisolone and 21-aminosteroids on mitogen-induced interleukin-6 and tumor necrosis factor-alpha production in human peripheral blood mononuclear cells. *J Pharmacol Exp Ther.* **275**, 850-3 (1995).

Cannon, G., Openshaw, SJ, Hibbs JB Jr, Hoidal JR, Huecksteadt TP, Griffiths MM. Nitric oxide production during adjuvant-induced and collagen-induced arthritis. *Arthritis Rheum.* **39**, 1677-84. (1996).

Cantoni, L., Rossi C, Rizzardini M, Gadina M, Ghezzi P. Interleukin-1 and tumour necrosis factor induce hepatic haem oxygenase. Feedback regulation by glucocorticoids. *Biochem J.* **279**, 891-4 (1991).

Cato, A., Wade, E. Molecular mechanisms of anti-inflammatory action of glucocorticoids. **18**, 371-8. (1996).

Ceponis, A., Konttinen YT, MacKevicius Z, Solovieva SA, Hukkanen M, Tamulaitiene M, Matulis A, Santavirta S. Aberrant vascularity and von Willebrand factor distribution in inflamed synovial membrane. *J Rheumatol.* **23**, 1880-6 (1996).

Cernanec, J., Guilak, F, Weinberg JB, Pisetsky DS, Fermor B. Influence of hypoxia and reoxygenation on cytokine-induced production of proinflammatory mediators in articular cartilage. *Arthritis Rheum* **46**, 968-75 (2002).

Chang, Y., Pearson CM, Abe C. Adjuvant polyarthritis. IV. Induction by a synthetic adjuvant: immunologic, histopathologic, and other studies. *Arthritis Rheum* **23**, 62-71 (1980).

Chapman, J. Measurement of tumour hypoxia by invasive and non-invasive procedures a review of recent clinical studies. *Radiotherapy and Oncol* **20**, 13-20 (1991).

Chen, C., Rosenbloom CL, Anderson DC, Manning AM. Selective inhibition of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 expression by inhibitors of I kappa. *J Immunol* **155**, 3538-45 (1995).

Chen, C., Pore, N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. *J Biol Chem.* **276**, 9519-25 (2001).

Chen, Z., Fisher, RJ, Riggs CW, Rhim JS, Lautenberger JA. Inhibition of vascular endothelial growth factor-induced endothelial cell migration by ETS1 antisense oligonucleotides. *Cancer Res* **57**, 2013-9 (1997).

Claxson, A., Grootveld, M, Chander C, Earl J, Haycock P, Mantle M, Williams SR, Silwood CJ, Blake DR. Examination of the metabolic status of rat air pouch inflammatory exudate by high field proton NMR spectroscopy. *Biochim Biophys Acta.* **1454**, 57-70. (1999).

Cline, J., Thrall, DE, Page RL, Franko AJ, Raleigh JA. Immunohistochemical detection of a hypoxia marker in spontaneous canine tumours. *Br J Cancer.* **62**, 925-31. (1990).

Clive, D., Stoff JS. Renal syndromes associated with nonsteroidal antiinflammatory drugs. *N Engl J Med.* **310**, 563-72 (1984).

Cohen, I., Holoshitz J, van Eden W, Frenkel A. T lymphocyte clones illuminate pathogenesis and affect therapy of experimental arthritis. *Arthritis and Rheumatism* **28**, 841-5 (1985a).

Cohen, J., Vischer TL, Carquin J, Blanchard F. A subset of rheumatoid arthritis patients with a pattern of Epstein-Barr virus antibodies similar to that found in primary and secondary immunodeficiency diseases. *Arthritis and Rheumatism* **28**, 339-40 (1985b).

Collins, T., Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *FASEB J* **9**, 899-909 (1995).

Colville-Nash PR, S. D. Angiogenesis and rheumatoid arthritis: pathogenic and therapeutic implications. *Ann Rheum Dis* **51**, 919-25 (1992).

Criscione, L., St Clair EW. Tumor necrosis factor-alpha antagonists for the treatment of rheumatic diseases. *Curr Opin Rheumatol.* **14**, 204-11 (2002).

Cush, J., Lipsky, PE. Cellular basis for rheumatoid inflammation. *Clinical Orthopaedics and Related Research* **265**, 9-22 (1991).

Czuwara-Ladykowska, J., Sementchenko VI, Watson DK, Trojanowska M. Ets1 is an effector of the transforming growth factor beta (TGF-beta) signaling pathway and an antagonist of the profibrotic effects of TGF-beta. *J Biol Chem.* **277**, 20399-408 (2002).

Dachs, G., Patterson AV, Firth JD, Ratcliffe PJ, Townsend KM, Stratford IJ, et al. Targeting gene expression to hypoxic tumor cells. *Nat Med* **3**, 515-20 (1997).

Dai, L., Zhang, Z, Winyard PG, Gaffney K, Jones H, Blake DR, Morris CJ. A modified form of low-density lipoprotein with increased electronegative charge is present in rheumatoid arthritis synovial fluid. *Free Radic Biol Med* **22**, 705-10 (1997).

Dai, L., Claxson, A, Marklund SL, Feakins R, Yousaf N, Chernajovsky Y, Winyard PG. Amelioration of antigen-induced arthritis in rats by transfer of extracellular superoxide dismutase and catalase genes. *Gene Ther.* **10**, 550-8. (2003).

De Gendt, C., De Clerck LS, Bridts CH, Van Osselaer N, Stevens WJ. Relationship between interleukin-8 and neutrophil adhesion molecules in rheumatoid arthritis. *Rheumatol Int* **16**, 169-73 (1996).

de Graeff-Meeder, E., Voorhorst M, van Eden W, Schuurman HJ, Huber J, Barkley D, Maini RN, Kuis W, Rijkers GT, Zegers BJ. Antibodies to the mycobacterial 65-kd heat-shock protein are reactive with synovial tissue of adjuvant arthritic rats and patients with rheumatoid arthritis and osteoarthritis. *Am J Pathol.* **137**, 1013-7 (1990).

deLeeuw, C. Tenth Nathalie Barr Lecture. Looking back, looking forward: thoughts along the journey. *J Hand Ther.* **9**, 6-9 (1996).

74. Dooley, S., Herlitzka, I, Hanselmann R, Ermis A, Henn W, Remberger K, Hopf T, Welter C. Constitutive expression of c-fos and c-jun, overexpression of ets-2, and reduced expression of metastasis suppressor gene nm23-H1 in rheumatoid arthritis. *Ann Rheum Dis.* **55**, 298-304. (1996).

Ebert, B., Firth,JD, Ratcliffe PJ. Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. *J Biol Chem.* **270**, 29083-9 (1995).

Edmonds, S., Blake DR, Morris CJ, Winyard PG. An imaginative approach to synovitis--the role of hypoxic reperfusion damage in arthritis. *J Rheumatol Suppl* **37**, 26-31 (1993).

Edwards, J. Synovial lining cell organization. *Scand J Rheumatol Suppl* **40**, 21-2 (1981).

Edwards, J. The origin of type A synovial lining cells. *Immunobiology* **161**, 227-31 (1982).

Edwards, J. The synovial lining - A moveable feast. *British Journal of Rheumatology* **28**, 534-36 (1989).

Egg, D. Concentrations of prostaglandins D2, E2, F2 alpha, 6-keto-F1 alpha and thromboxane B2 in synovial fluid from patients with inflammatory joint disorders and osteoarthritis. *Z Rheumatol* **43**, 89-96 (1984).

Elliott, M., Maini RN, Feldmann M, Kalden JR, Antoni C, Smolen JS, Leeb B, Breedveld FC, Macfarlane JD, Bijl H, et al. Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis. *Lancet*. **344**, 1105-10 (1994).

Ellis, L., Gilston, V, Soo CC, Morris CJ, Kidd BL, Winyard PG. Activation of the transcription factor NF-kappaB in the rat air pouch model of inflammation. *Ann Rheum Dis*. **59**, 303-7. (2000).

Emonard, H., Grimaud JA. Matrix metalloproteinases. *Cell Mol Biol* **36**, 131-53 (1990).

Endo, H., Akahoshi T, Takagishi K, Kashiwazaki S, Matsushima K. Elevation of interleukin-8 (IL-8) levels in joint fluids of patients with rheumatoid arthritis and the induction by IL-8 of leukocyte infiltration and synovitis in rabbit joints. *Lymphokine Cytokine Res* **10**, 245-52 (1991).

Evans, C., Robbins PD. The promise of a new clinical trial--intra-articular IL-1 receptor antagonist. *Proc Assoc Am Physicians*. **108**, 1-5 (1996).

Evans, C., Robbins PD. Gene therapy of arthritis. *Intern Med*. **38**, 233-9 (1999).

Evans, C., Ghivizzani SC, Herndon JH, Wasko MC, Reinecke J, Wehling P, Robbins PD. Clinical trials in the gene therapy of arthritis. *Clin Orthop*. **379 Suppl**, S300-7 (2000).

Fajardo, L., Kwan HH, Kowalski J, Prionas SD, Allison AC. Dual role of tumor necrosis factor-alpha in angiogenesis. *Am J Pathol* **140**, 539-44 (1992).

Falchuk, K., Goetzel, EJ, Kulka JP. Respiratory gases of synovial fluids. An approach to synovial tissue circulatory metabolic imbalance in rheumatoid arthritis. *The American Journal of Medicine* **499**, 223-231 (1970).

Farrell, I. A., Blake DR, Palmer RM, Moncada S. Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. *Ann Rheum Dis* **51**, 1219-22 (1992).

Fassbender, H. What destroys the joint in rheumatoid arthritis? *Arch Orthop Trauma Surg*. **117**, 2-7 (1998).

Feldmann, M. What is the mechanism of action of anti-tumour necrosis factor-alpha antibody in rheumatoid arthritis? *Int Arch Allergy Immunol*. **111**, 362-5 (1996).

Feldmann, M., Maini RN. Discovery of TNF-alpha as a therapeutic target in rheumatoid arthritis: preclinical and clinical studies. *Joint Bone Spine* **69**, 12-18 (2002).

Ferrara, N., Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev*. **18**, 4-25 (1997).

Ferreira, S., Nakamura, M. Prostaglandin hyperalgesia, a cAMP/Ca²⁺ dependent process. *Prostaglandins* **18**, 179-90. (1979).

Ferreira, S. Local control of inflammatory pain. *Agents Actions*. **11**, 636-8 (1981).

Finnegan, A., Kaplan CD, Cao Y, Eibel H, Glant TT, Zhang J. Collagen-induced arthritis is exacerbated in IL-10-deficient mice. *Arthritis Res Ther*. **5**, R18-24 (2003).

Firestein, G., Zvaifler NJ. How important are T-cells in chronic rheumatoid synovitis? *Arthritis and Rheumatism* **33**, 768-773 (1990).

Firestein, G., Alvaro-Gracia JM, Maki R, Alvaro-Garcia JM. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J Immunol* **144**, 3347-53 (1990).

Firestein, G. Invasive fibroblast-like synoviocytes in RA. Passive responders or transformed aggressors? *Arthritis and Rheumatism*. **39**, 1781-90 (1996).

Firestein, G. *Rheumatoid Arthritis - New frontiers in Pathogenesis and Treatment*. (ed. Firestein, G., Panayi, GS, Wollheim FA.) (Oxford University Press, 2000).

Firestein, G., Zvaifler NJ. How important are T cells in chronic rheumatoid synovitis?: II. T cell-independent mechanisms from beginning to end. *Arthritis and Rheumatism* **46**, 298-308 (2002).

Firestein, G. Evolving concepts of rheumatoid arthritis. *Nature* **423**, 356-60 (2003).

FitzGerald, O., Soden M, Yanni G, Robinson R, Bresnihan B. Morphometric analysis of blood vessels in synovial membranes obtained from clinically affected and unaffected knee joints of patients with rheumatoid arthritis. *Ann Rheum Dis* **50**, 792-6 (1991).

FitzGerald, O., Bresnihan B. Synovial membrane cellularity and vascularity. *Annals of the Rheumatic Diseases* **54**, 511-515 (1995).

Foresti, R., Clark JE, Green CJ, Motterlini R. Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxynitrite anions. *J Biol Chem* **272**, 18411-7 (1997).

Fox, D. Biological therapies: a novel approach to the treatment of autoimmune disease. *Am J Med. A* **99**, 82-8 (1995).

Foxwell, B., Browne K, Bondeson J, Clarke C, de Martin R, Brennan F, Feldmann M. Efficient adenoviral infection with IkappaB alpha reveals that macrophage tumor necrosis factor alpha production in rheumatoid arthritis is NF-kappaB dependent. *Proc Natl Acad Sci U S A*. **95**, 8211-5 (1998).

Franz, J., Pap T, Hummel KM, Nawrath M, Aicher WK, Shigeyama Y, Muller-Ladner U, Gay RE, Gay S. Expression of sentrin, a novel antiapoptotic

molecule, at sites of synovial invasion in rheumatoid arthritis. *Arthritis and Rheumatism* **42**, 599-607 (2000).

Frears, E., Zhang, Z, Blake DR, O'Connell JP, Winyard PG. Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. *FEBS Lett.* **381**, 21-4. (1996).

Frewin, D., Cleland LG, Jonsson JR, Robertson PW. Histamine levels in human synovial fluid. *J Rheumatol.* **13**, 13-4 (1986).

Friedl, H., Till GO, Ryan US, Ward PA. Mediator-induced activation of xanthine oxidase in endothelial cells. *FASEB J.* **3**, 2512--8 (1989).

Gaffney, K., Williams RB, Jolliffe VA, Blake DR. Intra-articular pressure changes in rheumatoid and normal peripheral joints. *Ann Rheum Dis* **54**, 670-3 (1995).

Geborek, P., Saxne, T, Pettersson H, Wollheim FA. Synovial fluid acidosis correlates with radiological joint destruction in rheumatoid arthritis knee joints. *J Rheumatol.* **16**, 468-72 (1989).

Gendi, N., Gibson K, Wordsworth BP. Effect of HLA type and hypocomplementaemia on the expression of parvovirus arthritis: one year follow up of an outbreak. *Ann Rheum Dis* **55**, 63-5 (1996).

Gerard, H., Wang Z, Wang GF, El-Gabalawy H, Goldbach-Mansky R, Li Y, Majeed W, Zhang H, Ngai N, Hudson AP, Schumacher HR. Chromosomal DNA from a variety of bacterial species is present in synovial tissue from patients with various forms of arthritis. *Arthritis and Rheumatism* **44**, 1689-97 (2001).

Ghadially, F. Ultrastructure of normal and torn menisci of the human knee joint. *Journal of Anatomy.* **136**, 773-91. (1983).

Ghivizzani, S., Lechman ER, Kang R, Tio C, Kolls J, Evans CH, Robbins PD. Direct adenovirus-mediated gene transfer of interleukin 1 and tumor necrosis factor alpha soluble receptors to rabbit knees with experimental arthritis has local and distal anti-arthritic effects. *Proc Natl Acad Sci U S A.* **95**, 4613-8 (1998).

Ghivizzani, S., Oligino TJ, Glorioso JC, Robbins PD, Evans CH. Direct gene delivery strategies for the treatment of rheumatoid arthritis. *Drug Discov Today.* **6**, 259-267 (2001).

Giatromanolaki, A., Harris AL. Tumour hypoxia, hypoxia signaling pathways and hypoxia inducible factor expression in human cancer. *Anticancer Res* **21**, 317-24 (2001).

Giatromanolaki, A., Sivridis E, Maltezos E, Athanassou N, Papazoglou D, Gatter KC, & Harris AL, K. M. Upregulated hypoxia inducible factor-1alpha and -2alpha pathway in rheumatoid arthritis and osteoarthritis. *Arthritis Res Ther.* **5**, R193-201 (2003).

Gilston, V., Jones HW, Soo CC, Coumbe A, Blades S, Kaltschmidt C, Baeuerle PA, Morris CJ, Blake DR, Winyard PG. NF-kappa B activation in human knee-joint synovial tissue during the early stage of joint inflammation. *Biochem Soc Trans* **25**, 812-6 (1997).

Godber, B., Doel, JJ, Durgan J, Eisenthal R, Harrison R. A new route to peroxynitrite: a role for xanthine oxidoreductase. *FEBS Lett.* **475**, 93-6. (2000).

Goddard, D., Grossman SL, Williams WV, Weiner DB, Gross JL, Eidsvoog K, Dasch JR. Regulation of synovial cell growth. Coexpression of transforming growth factor beta and basic fibroblast growth factor by cultured synovial cells. *Arthritis and Rheumatism* **35**, 1296-303 (1992).

Goldring, S., Gravallesse EM. Pathogenesis of bone erosions in rheumatoid arthritis. *Curr Opin Rheumatol* **12**, 195-9 (2000).

Goldring, S. Bone and joint destruction in rheumatoid arthritis: what is really happening? *J Rheumatol Suppl* **65**, 44-8 (2002).

Gomez, D., Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol* **74**, 111-22 (1997).

Grabowski, P., Wright, PK, Van 't Hof RJ, Helfrich MH, Ohshima H, Ralston SH. Immunolocalization of inducible nitric oxide synthase in synovium and cartilage in rheumatoid arthritis and osteoarthritis. *Br J Rheumatol.* **36**, 651-5 (1997).

Grabstein, K., Dower, S, Gillis S, Urdal D, Larsen A. Expression of interleukin 2, interferon-gamma, and the IL 2 receptor by human peripheral blood lymphocytes. *J Immunol.* **136**, 4503-8. (1986).

Granger, D., Hollwarth, ME, Parks DA. Ischemia-reperfusion injury: role of oxygen-derived free radicals. *Acta Physiol Scand Suppl.* **548**, 47-63. (1986).

Griffiths, L., Binley K, Iqbal S, Kan O, Maxwell P, Ratcliffe P, et al. The macrophage - a novel system to deliver gene therapy to pathological hypoxia. *Gene Ther* **7**, 255-62 (2000).

Gross, S., Wolin MS. Nitric oxide: pathophysiological mechanisms. *Annu Rev Physiol* **57**, 737-69 (1995).

Gutierrez-Rodriguez, O. Thalidomide. A promising new treatment for rheumatoid arthritis. *Arthritis and Rheumatism* **27**, 1118-21 (1984).

Gutierrez-Rodriguez, O., Starusta-Bacal P, Gutierrez-Montes O. Treatment of refractory rheumatoid arthritis--the thalidomide experience. *J Rheumatol* **16**, 158-63 (1989).

Haddad, J. Oxygen-sensitive pro-inflammatory cytokines, apoptosis signaling and redox-responsive transcription factors in development and pathophysiology. *Cytokines Cell Mol Ther* **7**, 1-14 (2002).

Hadjigogos, K. The role of free radicals in the pathogenesis of rheumatoid arthritis. *Panminerva Med* **45**, 7-13 (2003).

Halliwell, B. Oxygen radicals, nitric oxide and human inflammatory joint disease. *Ann Rheum Dis* **54**, 505-10 (1995).

Han, M., Kim JS, Park BH, Kim JR, Hwang BY, Lee HY, Song EK, Yoo WH. NF-kappaB-dependent lymphocyte hyperadhesiveness to synovial fibroblasts by hypoxia and reoxygenation: potential role in rheumatoid arthritis. *J Leukoc Biol* **73**, 525-9 (2003).

Han, Z., Boyle, DL, Shi Y, Green DR, Firestein GS. Dominant-negative p53 mutations in rheumatoid arthritis. *Arthritis Rheum.* **42**, 1088-92. (1999).

Handel, M., McMorro LB, Gravallesse EM. Nuclear factor-kappa B in rheumatoid synovium. Localization of p50 and p65. *Arthritis and Rheumatism* **38**, 1762-70 (1995).

Hang, L., Theofilopoulos, AN, Dixon FJ. A spontaneous rheumatoid arthritis-like disease in MRL/l mice. *J Exp Med.* **155**, 1690-701. (1982).

Hansson, M., Asea, A, Ersson U, Hermodsson S, Hellstrand K. Induction of apoptosis in NK cells by monocyte-derived reactive oxygen metabolites. *J Immunol.* **156**, 42-7. (1996).

Haraoui, B., Strand V, Keystone E. Biologic agents in the treatment of rheumatoid arthritis. *Curr Pharm Biotechnol* **1**, 217-33 (2000).

Haraoui, B., Strand, V, Keystone E. Biologic agents in the treatment of rheumatoid arthritis. *Curr Pharm Biotechnol* **1**, 217-33 (2000).

Harris, E. J. Rheumatoid arthritis. Pathophysiology and implications for therapy. *N Engl J Med* **3;322**, 1277-89 (1990).

Harrison, R. Structure and function of xanthine oxidoreductase: where are we now? *Free Radic Biol Med.* **33**, 774-97. (2002).

Hellsten-Westling, Y. Immunohistochemical localization of xanthine oxidase in human cardiac and skeletal muscle. *Histochemistry.* **100**, 215-22. (1993).

Henderson, B., Glynn LE. Metabolic alterations in the synoviocytes in chronically inflamed knee joints in immune arthritis in the rabbit: comparison with rheumatoid arthritis. *Br J Exp Pathol* **62**, 27-33 (1981).

Henderson, B., Higgs GA. Synthesis of arachidonate oxidation products by synovial joint tissues during the development of chronic erosive arthritis. *Arthritis and Rheumatism* **30**, 1149-56 (1987).

Hessler, J., Morel DW, Lewis LJ, Chisolm GM. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. *Arteriosclerosis.* **3**, 215-22 (1983).

Hitchon, C., Wong K, Ma G, Reed J, Lyttle D, El-Gabalawy H. Hypoxia-induced production of stromal cell-derived factor 1 (CXCL12) and vascular endothelial growth factor by synovial fibroblasts. *Arthritis Rheum* **46**, 2587-97 (2002).

Hodgkiss, R., Parrick, J, Porssa M, Stratford MR. Bioreductive markers for hypoxic cells: 2-nitroimidazoles with biotinylated 1-substituents. *J Med Chem.* **37**, 4352-6. (1994).

Hodgkiss, R., Stratford, MR, Dennis MF, Hill SA. Pharmacokinetics and binding of the bioreductive probe for hypoxia, NITP: effect of route of administration. *Br J Cancer.* **72**, 1462-8. (1995).

Hollander, A., Corke KP, Freemont AJ, Lewis CE. Expression of hypoxia-inducible factor 1alpha by macrophages in the rheumatoid synovium: implications for targeting of therapeutic genes to the inflamed joint. *Arthritis and Rheumatism* **44**, 1540-4 (2001).

Horwood, N., Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT. Activated T lymphocytes support osteoclast formation in vitro. *Biochem Biophys Res Commun* **265**, 144-50 (1999).

Hosaka, S., Akahoshi T, Wada C, Kondo H. Expression of the chemokine superfamily in rheumatoid arthritis. *Clin Exp Immunol* **97**, 451-7 (1994).

Hou, H., Grinberg, OY, Taie S, Leichtweis S, Miyake M, Grinberg S, Xie H, Csete M, Swartz HM. Electron paramagnetic resonance assessment of brain tissue oxygen tension in anesthetized rats. *Anesth Analg.* **96**, 1467-72, (2003).

Huizinga, T., Dijkmans BA, van der Velde EA, van de Pouw Kraan TC, Verweij CL, Breedveld FC. An open study of pentoxifylline and thalidomide as adjuvant therapy in the treatment of rheumatoid arthritis. *Ann Rheum Dis* **55**, 833-6 (1996).

Iwakura, Y., Tosu M, Yoshida E, Takiguchi M, Sato K, Kitajima I, Nishioka K, Yamamoto K, Takeda T, Hatanaka M, et al. Induction of inflammatory arthropathy resembling rheumatoid arthritis in mice transgenic for HTLV-I. *Science* **253**, 1026-8 (1991).

Iwasaka, C., Tanaka, K, Abe M, Sato Y. Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells. *J Cell Physiol.* **169**, 522-31. (1996).

Jackson, J., Minton, JA, Ho ML, Wei N, Winkler JD. Expression of vascular endothelial growth factor in synovial fibroblasts is induced by hypoxia and interleukin 1beta. *J Rheumatol.* **24**, 1253-9 (1997).

Jaffar, M., Williams KJ, Stratford IJ. Bioreductive and gene therapy approaches to hypoxic diseases. *Adv Drug Deliv Rev.* **53**, 217-28 (2001).

James, M., Cleland, LG, Rofe AM, Leslie AL. Intraarticular pressure and the relationship between synovial perfusion and metabolic demand. *J Rheumatol.* **17**, 521-7. (1990).

James, M., Cleland, LG, Rofe AM. Determinants of synovial fluid lactate concentration. *The Journal of Rheumatology* **19**, 1107-1110. (1992).

Jarasch, E., Bruder G, Heid HW. Significance of xanthine oxidase in capillary endothelial cells. *Acta Physiol Scand Suppl* **548**, 39-46 (1986).

Jayson, G., Zweit J, Jackson A, Mulatero C, Julyan P, Ranson M, Broughton L, Wagstaff J, Hakansson L, Groenewegen G, Bailey J, Smith N, Hastings D, Lawrance J, Haroon H, Ward T, McGown AT, Tang M, Levitt D, Marreaud S, Lehmann FF, Herold M, Zwierzina. Molecular imaging and biological evaluation of HuMV833 anti-VEGF antibody: implications for trial design of antiangiogenic antibodies. *J Natl Cancer Inst.* **94**, 1484-93 (2002).

Jayson, M., Dixon AS. Intra-articular pressure in rheumatoid arthritis of the knee. 3. Pressure changes during joint use. *Ann Rheum Dis* **29**, 401-8 (1970).

Jiang, B., Semenza, GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over physiologically relevant range of O₂ tension. *Am. J. Physiol.* **271**, C1172-180. (1996).

Jiang, B., Rue E, Wang GL, Roe R, Semenza GL. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J Biol Chem* **271**, 17771-8 (1996).

Johnson, L., Dyer R, Hupe DJ. Matrix metalloproteinases. *Curr Opin Chem Biol* **2**, 466-71 (1998).

Jones, M., Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ, Tarnawski AS. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. *Nat. Med.* **5**, 1348-9 (1999).

Jorgensen, C., Noel D, Apparailly F, Sany J. Stem cells for repair of cartilage and bone: the next challenge in osteoarthritis and rheumatoid arthritis. *Ann Rheum Dis* **60**, 305-9 (2001).

Karakurum, M., Shreeniwas R, Chen J, Pinsky D, Yan SD, Anderson M, Sunouchi K, Major J, Hamilton T, Kuwabara K, et al. Hypoxic induction of interleukin-8 gene expression in human endothelial cells. *J Clin Invest.* **93**, 1564-70 (1994).

Keesal, N., Wasserman MJ, Bookman A, Lapp V, Weber DA, Keystone EC. Thalidomide in the treatment of refractory rheumatoid arthritis. *J Rheumatol* **26**, 2344-7 (1999).

Kennedy, A., Raleigh, JA, Perez GM, Calkins DP, Thrall DE, Novotny DB, Varia MA. Proliferation and hypoxia in human squamous cell carcinoma of the cervix: first report of combined immunohistochemical assays. *Int J Radiat Oncol Biol Phys.* **37**, 897-905 (1997).

Keyse, S., Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci U S A* **86**, 99-103 (1989).

Kim, I., Lemmon MJ, Brown JM. The influence of irradiation of the tumor bed on tumor hypoxia: measurements by radiation response, oxygen electrodes, and nitroimidazole binding. *Radiat Res* **135**, 411-7 (1993).

Kitasato, H., Noda, M, Akahoshi T, Okamoto R, Koshino T, Murakami Y, Inoue M, Kawai S. Activated Ras modifies the proliferative response of rheumatoid synovial cells to TNF-alpha and TGF-alpha. *Inflamm Res.* **50**, 592-7. (2001).

Klasen, I., Melief MJ, Swaak TJ, Severijnen AJ, Hazenberg MP. Responses of synovial fluid and peripheral blood mononuclear cells bacterial antigens and autologous antigen presenting cells. *Ann Rheum Dis.* **52**, 127-32 (1993).

Klemp, P. Errors in the diagnosis and management of rheumatoid arthritis. *South African Journal of Cont. Med. Edu.* **1** (1983).

Klimiuk, P., Sierakowski S, Latosiewicz R, Cylwik B, Skowronski J, Chwiecko J. Serum matrix metalloproteinases and tissue inhibitors of metalloproteinases in different histological variants of rheumatoid synovitis. *Rheumatology (Oxford)* **41**, 78-87 (2002).

Kobayashi, I., Ziff, M. Electron microscopic studies of lymphoid cells in the rheumatoid synovial membrane. *Arthritis and Rheumatism* **16**, 471-486 (1973).

Koch, A., Polverini PJ, Leibovich SJ. Stimulation of neovascularization by human rheumatoid synovial tissue macrophages. *Arthritis and Rheumatism* **29**, 471-9 (1986).

Koch, A., Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elnor VM, Elnor SG, Strieter RM. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* **258**, 1798-801 (1992).

Koch, A., Friedman, J, Burrows JC, Haines GK, Bouck NP. Localization of the angiogenesis inhibitor thrombospondin in human synovial tissues. *Pathobiology*. **61**, 1-6. (1993).

Koch, A., Harlow LA, Haines GK, Amento EP, Unemori EN, Wong WL, Pope RM, & N., F. Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. *J Immunol* **152**, 4149-56 (1994).

Koga, S., Ogawa S, Kuwabara K, Brett J, Leavy JA, Ryan J, Koga Y, Plocinski J, Benjamin W, Burns DK, et al. Synthesis and release of interleukin 1 by reoxygenated human mononuclear phagocytes. *J Clin Invest*. **90**, 1007-15 (1992).

Kooij, A., Bosch, KS, Frederiks WM, Van Noorden CJ. High levels of xanthine oxidoreductase in rat endothelial, epithelial and connective tissue cells. A relation between localization and function? *Virchows Arch B Cell Pathol Incl Mol Pathol*. **62**, 143-50. (1992).

Koong, A., Chen EY, Giaccia AJ. Hypoxia causes the activation of nuclear factor kappa B through the phosphorylation of I kappa B alpha on tyrosine residues. *Cancer Res*. **54**, 1425-30 (1994).

Koppenol, W. The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radic Biol Med* **25**, 385-91 (1998).

Koppenol, W. Chemistry of peroxynitrite and its relevance to biological systems. *Met Ions Biol Syst*. **36**, 597-619 (1999).

Krause, A., Kamradt T, Burmester GR. Potential infectious agents in the induction of arthritides. *Rheumatol* **8**, 203-9 (1996).

Kruijsen, M., van den Berg, WB and van de Putte LBA. Sequential alterations of periarticular structures in antigen-induced arthritis in mice.

Histological observations on fibrous capsule, ligaments, bone and muscles, using whole joint sections. *British Journal of Experimental Pathology* **64**, 298-305 (1983).

Kung, A., Wang S, Klco JM, Kaelin WG, Livingston DM. Suppression of tumor growth through disruption of hypoxia-inducible transcription. *Nat Med* **6**, 1335-40 (2000).

Lafyatis, R., Remmers, EF, Roberts AB, Yocum DE, Sporn MB, Wilder RL. Anchorage-independent growth of synoviocytes from arthritic and normal joints. Stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. *J Clin Invest.* **83**, 1267-76. (1989).

Lavrovsky, Y., Schwartzman ML, Levere RD, Kappas A, Abraham NG. Identification of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. *Proc Natl Acad Sci U S A* **91**, 5987-91 (1994).

Lawrence, J., Valkenburg HA, Bremner JM, Ball J. Rheumatoid factors in families. *Ann Rheum Dis.* **29**, 269-74 (1970).

Lawrence, R., Hochberg MC, Kelsey JL, McDuffie FC, Medsger TA Jr, Felts & WR, S. L. Estimates of the prevalence of selected arthritic and musculoskeletal diseases in the United States. *J Rheumatol.* **16**, 427-41 (1989).

Lei, H., Grinberg, O, Nwaigwe, CI, Hou HG, Williams H, Swartz HM, Dunn JF. The effects of ketamine-xylazine anesthesia on cerebral blood flow and oxygenation observed using nuclear magnetic resonance perfusion imaging and electron paramagnetic resonance oximetry. *Brain Res.* **913**, 174-9. (2001).

Lelievre, E., Lionneton F, Soncin F, Vandenbunder B. The Ets family contains transcriptional activators and repressors involved in angiogenesis. *Int J Biochem Cell Biol.* **33**, 391-407 (2001).

Li, P., Sanz, I, O'Keefe RJ, Schwarz EM (). NF-kappa B regulates VCAM-1 expression on fibroblast-like synoviocytes. *J Immunol.* **164**, 5990-7 (2000).

Lopez-Guerrero, J., Ortiz MA, Paez E, Bernabeu C, Lopez-Bote JP. Therapeutic effect of recombinant vaccinia virus expressing the 60-kd heat-shock protein on adjuvant arthritis. *Arthritis Rheum* **37**, 1462-7 (1994).

Lund-Olesen, K. Oxygen tension in synovial fluids. *Arthritis and Rheumatism* **13**, 769-776 (1970).

Lunec, J., Halloran SP, White AG, Dormandy TL. Free-radical oxidation (peroxidation) products in serum and synovial fluid in rheumatoid arthritis. *J Rheumatol* **8**, 233-45 (1981).

Lydyard, P., Edwards JC. The pathophysiology of rheumatoid arthritis. *Clin Exp Rheumatol* **12**, S55-8 (1994).

MacGregor, A., Bamber S, Silman AJ. A comparison of the performance of different methods of disease classification for rheumatoid arthritis. Results of an analysis from a nationwide twin study. *J Rheumatol.* **21**, 1420-6 (1994).

MacGregor, A., Snieder H, Rigby AS, Koskenvuo M, Kaprio J, Aho K, Silman AJ. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis Rheum* **43**, 30-7 (2000).

Maines, M. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J.* **2**, 2557-68 (1988).

Maini, R. Exploring immune pathways in rheumatoid arthritis. *British Journal of Rheumatology* **28**, 466-79 (1995).

Maini, R., Breedveld FC, Kalden JR, Smolen JS, Davis D, Macfarlane JD, Antoni C, Leeb B, Elliott MJ, Woody JN, Schaible TF, Feldmann M. Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis and Rheumatism* **41**, 1552-63 (1998).

Makarov, S., Olsen JC, Johnston WN, Anderle SK, Brown RR, Baldwin AS Jr, Haskill JS, Schwab JH. Suppression of experimental arthritis by gene transfer of interleukin 1 receptor antagonist cDNA. *Pro Natl Accad Sci USA.* **93**, 402-6 (1996).

Makarov, S. NF-kappa B in rheumatoid arthritis: a pivotal regulator of inflammation, hyperplasia, and tissue destruction. *Arthritis Res.* **3**, 200-6. (2001).

Manley, P., Martiny-Baron G, Schlaeppi JM, Wood JM. Therapies directed at vascular endothelial growth factor. *Expert Opin Investig Drugs.* **11**, 1715-36 (2002).

Manning, A., Bell FP, Rosenbloom CL, Chosay JG, Simmons CA, Northrup JL, Shebuski RJ, Dunn CJ, Anderson DC. NF-kappa B is activated during acute inflammation in vivo in association with elevated endothelial cell adhesion molecule gene expression and leukocyte recruitment. *J Inflamm* **45**, 283-96 (1995).

Mapp, P., Kidd BL, Gibson SJ, Terry JM, Revell PA, Ibrahim NB, Blake DR, Polak JM. Substance P-, calcitonin gene-related peptide- and C-flanking peptide of neuropeptide Y-immunoreactive fibres are present in normal synovium but depleted in patients with rheumatoid arthritis. *Neuroscience* **37**, 143-53 (1990).

Mapp, P., Grootveld MC, Blake DR. Hypoxia, oxidative stress and rheumatoid arthritis. *Br Med Bull* **51**, 419-36 (1995).

Mapp, P., Klocke, R, Walsh DA, Chana JK, Stevens CR, Gallagher PJ, Blake DR. Localization of 3-nitrotyrosine to rheumatoid and normal synovium. *Arthritis Rheum.* **44**, 1534-9. (2001).

Marok, R., Winyard, PG, Coumbe A, Kus ML, Gaffney K, Blades S, Mapp PI, Morris CJ, Blake DR, Kaltschmidt C, Baeuerle PA. Activation of the transcription factor nuclear factor-kappaB in human inflamed synovial tissue. *Arthritis Rheum.* **39**, 583-91 (1996).

Massova, I., Kotra LP, Mobashery S. Structural insight into the binding motifs for the calcium ion and the non-catalytic zinc in matrix metalloproteases. *Bioorg Med Chem Lett.* **8**, 853-8 (1998).

Matsumoto, S., Muller-Lander U, Gay RE, Nishioka K, Gay S. Ultrastructural demonstration of apoptosis, Fas and Bcl-2 expression of rheumatoid synovial fibroblasts. *J Rheumatol* **23**, 1345-52. (1996).

Matsuno, H., Yudoh K, Uzuki M, Nakazawa F, Sawai T, Yamaguchi N, Olsen BR, Kimura T. Treatment with the angiogenesis inhibitor endostatin: a novel therapy in rheumatoid arthritis. *J Rheumatol* **29**, 890-5 (2002).

McCall, T., Palmer RM, Moncada S. Induction of nitric oxide synthase in rat peritoneal neutrophils and its inhibition by dexamethasone. *Eur J Immunol* **21**, 2523-7 (1991).

McGough, K., Jackson JR, Minton JA, Marshall LA, Jacobs RS, Winkler JD. Inflammatory PGE2 production is maintained during hypoxia in rheumatoid synovial fibroblasts. *Inflamm Res* **46**, S147-8 (1997).

McInnes, I., Leung BP, Field M, Wei XQ, Huang FP, Sturrock RD, Kinninmonth A, Weidner J, Mumford R, Liew FY,. Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. *J Exp Med* **184**, 1519-24 (1996).

Melillo, G., Musso T, Sica A, Taylor LS, Cox GW, Varesio L. A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *J Exp Med* **182**, 1683-93 (1995).

Melillo, G., Musso T, Sica A, Taylor LS, Cox GW, Varesio L. A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *J Exp Med.* **182**, 1683-93. (1995).

Merry, P., Grootveld M, Lunec J, Blake DR. Oxidative damage to lipids within the inflamed human joint provides evidence of radical-mediated hypoxic-reperfusion injury. *Am J Clin Nutr* **53**, 362S-369S (1991).

Merry, P., William, R, Cox N, King JB, Blake DR. Comparative study of intra-articular pressure dynamics in joints with acute traumatic and chronic inflammatory effusions: potential implications for hypoxic-reperfusion injury. *Annals of the Rheumatic Diseases* **50**, 917-20 (1991).

Miesel, R., Zuber, M. Elevated levels of xanthine oxidase in serum of patients with inflammatory and autoimmune rheumatic diseases. *Inflammation.* **17**, 551-61 (1993).

Migita, K., Yamasaki, S, Ida H, Kita M, Hida A, Shibatomi K, Kawakami A, Aoyagi T, Eguchi K. The role of peroxynitrite in cyclooxygenase-2 expression of rheumatoid synovium. *Clin Exp Rheumatol.* **20**, 59-62. (2002).

Millar, T., Stevens CR, Benjamin N, Eisenthal R, Harrison R, Blake DR. Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. *FEBS Lett* **427**, 225-8 (1998).

Miller, G., Best, MW, Franko AJ, Koch CJ, Raleigh JA. Quantitation of hypoxia in multicellular spheroids by video image analysis. *Int J Radiat Oncol Biol Phys* **16**, 949-52. (1989).

Mitchell, L., Decarie D, Shukin R, Tingle AJ, Ford DK, Lacroix M, Zrein M. Cellular hyperimmunoreactivity to rubella virus synthetic peptides in chronic rubella associated arthritis. *Ann Rheum Dis* **52**, 590-4 (1993).

Mitrovic, D. The mechanism of cartilage destruction in rheumatoid arthritis. *Arthritis and Rheumatism* **28**, 1192-1193 (1985).

Modlin, C., Todd GT, Cohen TD, Fairchild RL. Transfer of endogenous retroviral superantigen from donor to recipient B cells following priming to induce peripheral T cell tolerance. *Cell Immunol* **164**, 217-26 (1995).

Mohan, N., Edwards ET, Cupps TR, Oliverio PJ, Sandberg G, Crayton H, Richert JR, Siegel JN. Demyelination occurring during anti-tumor necrosis factor alpha therapy for inflammatory arthritides. *Arthritis Rheum.* **44**, 2862-9 (2001).

Morales-Ducret, J., Wayner, E, Elices MJ, Alvaro-Gracia JM, Zvaifler NJ, Firestein GS. Alpha 4/beta 1 integrin (VLA-4) ligands in arthritis. Vascular cell adhesion molecule-1 expression in synovium and on fibroblast-like synoviocytes. *J Immunol.* **149**, 1424-31 (1992).

Morel, D., Hessler JR, Chisolm GM. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J Lipid Res* **24**, 1070-6 (1983).

Morgan, M., Suttmuller RP, Witteveen HJ, van Duivenvoorde LM, Zanelli E, Melief CJ, Snijders A, Offringa R, de Vries RR, Toes RE. CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis. *Arthritis Rheum* **48**, 1452-60 (2003).

Morris, C., Earl JR, Trenam CW, Blake DR. Reactive oxygen species and iron--a dangerous partnership in inflammation. *Int J Biochem Cell Biol* **27**, 109-22 (1995).

Muller-Ladner, U. T cell-independent cellular pathways of rheumatoid joint destruction. *Rheumatol* **7**, 222-8 (1995).

Muller-Ladner, U., Kriegsmann, J, Franklin BN, Matsumoto S, Geiler T, Gay RE and Gay S. Synovial fibroblasts of patients with rheumatoid arthritis attach to

and invade normal human cartilage when engrafted into SCID mice. *American Journal of Pathology* **149**, 1607-1615. (1996).

Murrell, G., Francis MJ, Bromley L. Modulation of fibroblast proliferation by oxygen free radicals. *Biochem J* **265**, 659-65 (1990).

Murrell, G., Jang D, Williams RJ. Nitric oxide activates metalloprotease enzymes in articular cartilage. *Biochem Biophys Res Commun.* **5;206**, 15-21 (1995).

Nagase, H., Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem* **274**, 21491-4 (1999).

Nagashima, M., Yoshino S, Aono H, Takai M, Sasano M. Inhibitory effects of anti-rheumatic drugs on vascular endothelial growth factor in cultured rheumatoid synovial cells. *Clin Exp Immunol* **116**, 360-5 (1999).

Nakajima, T., Aono, H, Hasunuma T, Yamamoto K, Shirai T, Hirohata K, Nishioka K. Apoptosis and functional Fas antigen in rheumatoid arthritis synoviocytes. *Arthritis Rheum.* **38**, 485-91 (1995).

Nakamura, R., Asai H, Sonozaki H, Nagano M. Phenolsulphonphthalein clearance from the knee joint in normal and pathological states. *Ann Rheum Dis* **26**, 246-50 (1967).

Nakashima, M., Eguchi, K, Aoyagi T, Yamashita I, Ida H, Sakai M, Shimada H, Kawabe Y, Shigenobu N, Koji T and Nakane PK. Expression of basic fibroblast growth factor in synovial tissues from patients with rheumatoid arthritis: detection by immunological staining and in situ hybridisation. *Annals of the Rheumatic Diseases* **53**, 45-50 (1994).

Naughton, D., Haywood, R, Blake DR, Edmonds S, Hawkes GE and Grootveld M. A comparative evaluation of the metabolic profiles of normal and inflammatory knee-joint synovial fluids by high resolution proton NMR spectroscopy. *Federation of European Biochemical Societies.* **332**, 221-225 (1993a).

Naughton, D., Whelan, M, Smith EC, Williams R, Blake DR and Grootveld M. An investigation of the abnormal metabolic status of synovial fluid from patients with rheumatoid arthritis by high field proton nuclear magnetic resonance

spectroscopy. *Federation of European Biochemical Societies*. **317**, 135-138 (1993b).

Newbold, B. Chemotherapy of arthritis induced in rats by injection of mycobacterial adjuvant. *Br J Pharmacol* **21**, 127-136. (1963).

Nielsen, H., Thomsen, K, Eriksen EF, Charles P, Storm T, Mosekilde L. The effects of high-dose glucocorticoid administration on serum bone gamma carboxyglutamic acid-containing protein, serum alkaline phosphatase and vitamin D metabolites in normal subjects. *Bone Miner.* **4**, 105-13. (1988).

Nita, I., Ghivizzani SC, Galea-Lauri J, Bandara G, Georgescu HI, Robbins PD, Evans CH. Direct gene delivery to synovium. An evaluation of potential vectors in vitro and in vivo. *Arthritis and Rheumatism* **39**, 820-8 (1996).

Novotna, J., Herget J. Possible role of matrix metalloproteinases in reconstruction of peripheral pulmonary arteries induced by hypoxia. *Physiol Res* **51**, 323-34 (2002).

O'Brien, W., Bagby GF. Rare adverse reactions to nonsteroidal antiinflammatory drugs (3). *J Rheumatol* **12**, 562-7 (1985).

Oikawa, M., Abe M, Kurosawa H, Hida W, Shirato K, Sato Y. Hypoxia induces transcription factor ETS-1 via the activity of hypoxia-inducible factor-1. *Biochem Biophys Res Commun.* **289**, 39-43 (2001).

Okada, Y. Tumor cell-matrix interaction: pericellular matrix degradation and metastasis. *Verh Dtsch Ges Pathol* **84**, 33-42 (2000).

Okamoto, T., Akaike T, Nagano T, Miyajima S, Suga M, Ando M, Ichimori K, Maeda H. Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide. *Arch Biochem Biophys* **342**, 261-74 (1997).

Ossandon, A., Cassara EA, Priori R, Valesini G. Thalidomide: focus on its employment in rheumatologic diseases. *Clin Exp Rheumatol.* **20**, 709-18 (2002).

Otani, K., Nita I, Macaulay W, Georgescu HI, Robbins PD, Evans CH. Suppression of antigen-induced arthritis in rabbits by ex vivo gene therapy. *J Immunol* **156**, 3558-62 (1996).

Pablos, J., Santiago B, Galindo M, Torres C, Brehmer MT, Blanco FJ, Garcia-Lazaro FJ. Synoviocyte-derived CXCL12 is displayed on endothelium and induces angiogenesis in rheumatoid arthritis. *J Immunol* **170**, 2147-52 (2003).

Padyukov, L., Lampa J, Heimbürger M, Ernestam S, Cederholm T, Lundkvist I, Andersson P, Hermansson Y, Harju A, Klareskog L, Bratt J. Genetic markers for the efficacy of tumour necrosis factor blocking therapy in rheumatoid arthritis. *Ann Rheum Dis* **62**, 526-9 (2003).

Palmer, R., Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**, 524-6 (1987).

Pan, R., Chen SL, Xiao X, Liu DW, Peng HJ, Tsao YP. Therapy and prevention of arthritis by recombinant adeno-associated virus vector with delivery of interleukin-1 receptor antagonist. *Arthritis Rheum* **43**, 289-97 (2000).

Panayi, G. The pathogenesis of rheumatoid arthritis and the development of therapeutic strategies for the clinical investigation of biologics. *Inflammation : Mechanisms and Therapeutics* **47**, 1-21 (1995).

Panayi, G. T-cell-dependent pathways in rheumatoid arthritis. *Rheumatol* **9**, 236-40 (1997).

Panchenko, M., Farber HW, Korn JH. Induction of heme oxygenase-1 by hypoxia and free radicals in human dermal fibroblasts. *Am J Physiol Cell Physiol* **278**, C92-C101 (2000).

Pap, T., Muller-Ladner U, Gay RE, Gay S. Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. *Arthritis Res.* **2**, 361-7 (2002).

Patrignani, P., Capone, ML, Tacconelli S. Clinical pharmacology of etoricoxib: a novel selective COX2 inhibitor. *Expert Opin Pharmacother.* **4**, 265-84 (2003).

Peacock, D., Banquerigo ML, Brahn E. Angiogenesis inhibition suppresses collagen arthritis. *J Exp Med.* **175**, 1135-8 (1992).

Pearson, C., Waksman, BH, and Sharp JT. Studies on arthritis and other lesions induced in rats by mycobacterial adjuvant. Comparison of experimental

process with human disease. *Journal of Experimental Medicine* **113**, 485-491 (1961).

Pelegri, C., Franch, A, Castellote C and Castell M. Immunohistochemical changes in synovial tissue during the course of adjuvant arthritis. *The Journal of Rheumatology* **22**, 124-131 (1995).

Pogue, B., Paulsen KD, O'Hara JA, Wilmot CM, Swartz HM. Estimation of oxygen distribution in RIF-1 tumors by diffusion model-based interpretation of pimonidazole hypoxia and eppendorf measurements. *Radiat Res* **155**, 15-25 (1995).

Poss, W., Huecksteadt, TP, Panus PC, Freeman BA, Hoidal JR. Regulation of xanthine dehydrogenase and xanthine oxidase activity by hypoxia. *Am J Physiol.* **270**, L941-6. (1996).

Poutler, L., Duke, O, Hobbs S, Janossy G and Panayi G. Histochemical discrimination of HLA-DR positive cell populations in the normal and arthritic synovial lining. *Clinical and Experimental Immunology* **48**, 381-388. (1982).

Pozza, M., Guerra M, Manzini E, Calza L. A histochemical study of the rheumatoid synovium: focus on nitric oxide, nerve growth factor high affinity receptor, and innervation. *J Rheumatol* **27**, 1121-7 (2000).

Pybus, D., Torda TA. Dose-effect relationships of extradural morphine. *Br J Anaesth.* **54**, 1259-62 (1982).

Pybus, P. Osteoarthritis. A new neurological method of pain control. *Med Hypotheses.* **14**, 413-22 (1984).

Pybus, P. Metronidazole in rheumatoid arthritis. *S Afr Med J.* **67**, 1039-40 (1985).

Radi, R., Beckman, JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* **288**, 481-7 (1991).

Radi, R., Rodriguez M, Castro L, Telleri R. Inhibition of mitochondrial electron transport by peroxynitrite. *Arch Biochem Biophys* **308**, 89-95 (1994).

Ragno, S., Morris CJ, Coumbe A, Mapp PI, Colston MJ, Blake DR, Winrow VR. PPD and hsp65 induced monoarthritis initiates spontaneous recurrent flares in Lewis rats. *Ann Rheum Dis* **54**, 59-65 (1995).

Ragno, S., Colston MJ, Lowrie DB, Winrow VR, Blake DR, Tascon R. Protection of rats from adjuvant arthritis by immunization with naked DNA encoding for mycobacterial heat shock protein 65. *Arthritis Rheum* **40**, 277-83 (1997).

Rajagopalan, S., Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* **98**, 2572-9 (1996).

Rajpurohit, R., Koch, CJ, Tao Z, Teixeira CM, Shapiro IM. Adaptation of chondrocytes to low oxygen tension: relationship between hypoxia and cellular metabolism. *Journal of Cell Physiology* **168**, 424-432. (1996).

Raleigh, J., Miller, GG, Franko AJ, Koch CJ, Fuciarelli AF, Kelly DA. Fluorescence immunohistochemical detection of hypoxic cells in spheroids and tumours. *Br J Cancer*. **56**, 395-400. (1987).

Raleigh, J., Chou, SC, Arteel GE, Horsman MR. Comparisons among pimonidazole binding, oxygen electrode measurements, and radiation response in C3H mouse tumors. *Radiat Res*. **151**, 580-9 (1997).

Ralston, S., Ho LP, Helfrich MH, Grabowski PS, Johnston PW, Benjamin N. Nitric oxide: a cytokine-induced regulator of bone resorption. *J Bone Miner Res*. **10**, 1040-9 (1995).

Ralston, S. The Michael Mason Prize Essay 1997. Nitric oxide and bone: what a gas! *Br J Rheumatol*. **36**, 831-8 (1997).

Rashad, S., Revell P, Hemingway A, Low F, Rainsford K, Walker F. Effect of non-steroidal anti-inflammatory drugs on the course of osteoarthritis. *Lancet* . **2**, 519-22 (1989).

Ratcliffe, P., O'Rourke JF, Maxwell PH, Pugh CW. Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. *J Exp Biol* **201**, 1153 (1998).

Rawlinson, S., Zaman, G, Mosley JR, Pitsillides AA, Lanyon LE. Heme oxygenase isozymes in bone: induction of HO-1 mRNA following physiological levels of mechanical loading in vivo. *Bone*. **23**, 433-6 (1998).

Redlich, K., Kiener, HP, Schett G, Tohidast-Akrad M, Selzer E, Radda I, Stummvoll GH, Steiner CW, Groger M, Bitzan P, Zenz P, Smolen JS, Steiner G. Over expression of transcription factor Ets-1 in rheumatoid arthritis synovial membrane: regulation of expression and activation by interleukin-1 and tumor necrosis factor alpha. *Arthritis Rheum*. **44**, 266-74 (2001).

Reeves, G., Todd, I. *Lecture Notes on Immunology*. (Blackwell Scientific Publications, UK., 1991).

Remmers, E., Sano, H, Wilder RL. Platelet-derived growth factors and heparin-binding (fibroblast) growth factors in the synovial tissue pathology of rheumatoid arthritis. *Semin Arthritis Rheum*. **21**, 191-9. (1991).

Robbins, P., Evans CH, Chernajovsky Y. Gene therapy for arthritis. *Gene Ther*. **10**, 902-11 (2003).

Robinson, D., Tashjian, AH Jr, Levine L. Prostaglandin-stimulated bone resorption by rheumatoid synovia. A possible mechanism for bone destruction in rheumatoid arthritis. *J Clin Invest*. **56**, 1181-8. (1975).

Roessler, B., Allen ED, Wilson JM, Hartman JW, Davidson BL. Adenoviral-mediated gene transfer to rabbit synovium in vivo. *J Clin Invest*. **92**, 1085-92 (1993).

Roitt, I. *Essential Immunology*. (Blackwell Scientific Publications, UK, 1991).

Roivainen, A., Soderstrom KO, Pirila L, Aro H, Kortekangas P, Merilahti-Palo R, Yli-Jama T, Toivanen A, Toivanen P. Oncoprotein expression in human synovial tissue: an immunohistochemical study of different types of arthritis. *Br J Rheumatol* **35** (1996).

Rolett , E., Azzawi,A, Liu, KEJ, Yongbi MN, Swartz HM, Dunn JF. Critical oxygen tension in rat brain: a combined ³¹P-NMR and EPR oximetry study. *Am J Physiol Regulatory Integrative Comp Physiol*. **279**, R9–R16 (2000).

Rothschild, B., Masi, AT. Pathogenesis of rheumatoid arthritis. A vascular hypothesis. *Seminars in Arthritis and Rheumatism* **12**, 1445-1448. (1982).

Roudier, J., Petersen J, Rhodes GH, Luka J, Carson DA. Susceptibility to rheumatoid arthritis maps to a T-cell epitope shared by the HLA-Dw4 DR beta-1 chain and the Epstein-Barr virus glycoprotein gp110. *Proc Natl Acad Sci U S A* **86**, 5104-8 (1987).

Roudier, J., Petersen, J, Rhodes GH, Luka J and Carson DA. Susceptibility to rheumatoid arthritis maps to a T-cell epitope shared by the HLA-Dw4DR -1 chain and the epstein-barr virus glycoprotein gp 110. *Proceedings of the National Academy of Sciences of the USA*. **86** (1989).

Rowley, D., Gutteridge JM, Blake D, Farr M, Halliwell B. Lipid peroxidation in rheumatoid arthritis: thiobarbituric acid-reactive material and catalytic iron salts in synovial fluid from rheumatoid patients. *Clin Sci (Lond)*. **66**, 691-5 (1984).

Ruth, J., Volin MV, Haines GK 3rd, Woodruff DC, Katschke KJ Jr, Woods JM, Park CC, Morel JC, Koch AE. Fractalkine, a novel chemokine in rheumatoid arthritis and in rat adjuvant-induced arthritis. *Arthritis and Rheumatism* **44**, 1568-81 (2001).

Sakurai, H., Kohsaka H, Liu MF, Higashiyama H, Hirata Y, Kanno K, Saito I, Miyasaka N. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. *J Clin Invest* **96**, 2357-63 (1995).

Salceda, S., Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* **272**, 22642-7 (1997).

Salvemini, D., Masini E, Anggard E, Mannaioni PF, Vane J. Synthesis of a nitric oxide-like factor from L-arginine by rat serosal mast cells: stimulation of guanylate cyclase and inhibition of platelet aggregation. *Biochem Biophys Res Commun*. **169**, 590-601 (1990).

Salvemini, D., Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P. Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci U S A*. **90**, 7240-4 (1993).

Sandhu, J., Robertson, S, Birnboim HC, Goldstein R. Distribution of protein nitrotyrosine in synovial tissues of patients with rheumatoid arthritis and osteoarthritis. *J Rheumatol*. **30**, 1173-81. (2003).

Sano, H., Hla T, Maier JA, Crofford LJ, Case JP, Maciag T, Wilder RL. In vivo cyclooxygenase expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis. *J Clin Invest* **89**, 97-108 (1992).

Schauf, C., Moffett, DF, Moffett S. *Human Physiology: foundations and frontiers* (ed. Allen, D.) (Times Mirror/Mosby Company., 1990).

Schmedtje, J. J., Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J Biol Chem* **272**, 601-8 (1997).

Schmedtje, J. J., Ji YS. Hypoxia and Molecular Cardiovascular Medicine. *Trends Cardiovasc Med* **8**, 24-33 (1998).

Schreck, R., Albermann K, Baeuerle PA. Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic Res Commun* **17**, 221-37 (1992).

Schumacher, H., Kitridou, RC. Synovitis of recent onset. A clinicopathologic study during the first month of disease. *Arthritis and Rheumatism* **15**, 465-485 (1972).

Sedgwick, A., Moore, AR, Al-Duaij AY, Edwards JC, Willoughby DA. The immune response to pertussis in the 6-day air pouch: a model of chronic synovitis. *Br J Exp Pathol*. **66**, 455-64. (1985a.).

Sedgwick, A., Moore, AR, Al-Duaij AY, Edwards JC, Willoughby DA. Studies into the influence of carrageenan-induced inflammation on articular cartilage degradation using implantation into air pouches. *Br J Exp Pathol*. **66**, 445-53. (1985b.).

Seki, T., Selby J, Haupl T, Winchester R. Use of differential subtraction method to identify genes that characterize the phenotype of cultured rheumatoid arthritis synoviocytes. *Arthritis and Rheumatism* **41**, 1356-64 (1998).

Sekimoto, T., Hamada K, Oike Y, Matsuoka T, Matsumoto M, Chosa E, Suda T, Tajima N, Yamamura K. Effect of direct angiogenesis inhibition in rheumatoid arthritis using a soluble vascular endothelial growth factor receptor 1 chimeric protein. *J Rheumatol* **29**, 240-5 (2002).

Semenza, G. Regulation of mammalian O₂ homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* **15**, 551-78 (1999).

Semenza, G. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol* **88**, 1474-80 (2000a).

Semenza, G. Expression of hypoxia-inducible factor 1: mechanisms and consequences. *Biochem Pharmacol.* **59**, 47-53 (2000b).

Semenza, G. Oxygen-regulated transcription factors and their role in pulmonary disease. *Respir Res* **1**, 159-62 (2000b).

Semenza, G. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med* **7**, 345-50 (2001).

Sewell, K., Trentham DE. Pathogenesis of rheumatoid arthritis. *Lancet.* **341**, 283-6 (1993).

Shahrara, S., Volin MV, Connors MA, Haines GK, Koch AE. Differential expression of the angiogenic Tie receptor family in arthritic and normal synovial tissue. *Arthritis Res.* **4**, 201-8 (2002).

Shapiro, I., Mansfield, KD, Evans SM, Lord EM and Koch CJ. Chondrocytes in the endochondral growth cartilage are not hypoxic. *American Journal of Physiology* **272**, C1134-1143 (1997).

Shiozawa, S., Tanaka Y, Fujita T, Tokuhisa T. Destructive arthritis without lymphocyte infiltration in H2-c-fos transgenic mice. *J Immunol* **148**, 3100-4 (1992).

Shiozawa, S., Shimizu, K, Tanaka K and Hino K (). 99(6):. Studies on the contribution of c-fos/AP-1 to arthritic joint destruction. *Journal of Clinical Investigation* **99**, 1210-1216 (1997).

Shreeniwas, R., Koga S, Karakurum M, Pinsky D, Kaiser E, Brett J, Wolitzky BA, Norton C, Plocinski J, Benjamin W, et al. Hypoxia-mediated induction of endothelial cell interleukin-1 alpha. An autocrine mechanism promoting expression

of leukocyte adhesion molecules on the vessel surface. *J Clin Invest.* **90**, 2333-9 (1992).

Sieper, J., Kingsley GH, Marker-Hermann E. Aetiological agents and immune mechanisms in enterogenic reactive arthritis. *Baillieres Clin Rheumatol.* **10**, 105-21 (1996).

Sieper, J., Kingsley G. Recent advances in the pathogenesis of reactive arthritis. *Immunol Today* **17**, 160-3 (1996).

Silman, A., MacGregor AJ, Thomson W, Holligan S, Carthy D, Farhan A, Ollier WE. Twin concordance rates for rheumatoid arthritis: results from a nationwide study. *Br J Rheumatol* **32**, 903-7 (1993).

Silman, A., Pearson JE. Epidemiology and genetics of rheumatoid arthritis. *Arthritis Res.* **4**, S265-72 (2002).

Singh, D., Nazhat NB, Fairburn K, Sahinoglu T, Blake DR, Jones P. Electron spin resonance spectroscopic demonstration of the generation of reactive oxygen species by diseased human synovial tissue following ex vivo hypoxia-reoxygenation. *Ann Rheum Dis* **54**, 94-9 (1995).

Smith, W., Marnett LJ, DeWitt DL. Prostaglandin and thromboxane biosynthesis. *Pharmacol Ther* **49**, 153-79 (1991).

Soo, C. in *Department of Pharmacology* (University of London, 1999).

Steinbrech, D., Longaker MT, Mehrara BJ, Saadeh PB, Chin GS, Gerrets RP, Chau DC, Rowe NM, Gittes GK. Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation. *J Surg Res.* **84**, 127-33 (1999).

Steerk, H., Bielinski, TC and Budzilovich T. Chronic polyarthritis in rats injected with spleen in adjuvants. *American Journal of Pathology* **30**, 616-621. (1954).

Stevens, C., Blake, DR, Merry P, Revell RA and Levick JR. A comparative study by morphometry of the microvasculature in normal and rheumatoid synovium. *Arthritis and Rheumatism* **34**, 1509-1513 (1991a).

Stevens, C., Benboubetra M, Harrison R, Sahinoglu T, Smith EC, Blake DR. Localisation of xanthine oxidase to synovial endothelium. *Ann Rheum Dis* **50**, 760-2 (1991b).

Stocker, R., Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* **235**, 1043-6 (1987).

Storgard, C., Stupack, DG, Jonczyk A, Goodman SL, Fox RI, Cheresch DA. Decreased angiogenesis and arthritic disease in rabbits treated with an alphavbeta3 antagonist. *J Clin Invest.* **103**, 47-54. (1999).

Stratford, M., Clarke, ED, Hodgkiss RJ, Middleton RW, Wardman P. Nitroaryl compounds as potential fluorescent probes for hypoxia. II. Identification and properties of reductive metabolites. *Int J Radiat Oncol Biol Phys.* **10**, 1353-6. (1984).

Stuehr, D., Marletta MA. Synthesis of nitrite and nitrate in murine macrophage cell lines. *Cancer Res.* **47**, 5590-4 (1987).

Suda, T., Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* **20**, 345-57 (1999).

Sun, X., Kanwar JR, Leung E, Lehnert K, Wang D, Krissansen GW. Gene transfer of antisense hypoxia inducible factor-1 alpha enhances the therapeutic efficacy of cancer immunotherapy. *Gene Ther* **8**, 638-45 (2001).

Swartz, H., Walczak, T. Developing in vivo EPR oximetry for clinical use. *Adv Exp Med Biol.* **454**, 243-52. (1998).

Swartz, H. Measuring real levels of oxygen in vivo: opportunities and challenges. *Biochem Soc Trans.* **30**, 248-52. (2002).

Szekanecz, Z., Kim J, Koch AE. Chemokines and chemokine receptors in rheumatoid arthritis. *Semin Immunol* **15**, 15-21 (2003).

Tak, P., Smeets, TJ, Daha MR, Kluin PM, Meijers KA, Brand R, Meinders AE, Breedveld FC. Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity. *Arthritis Rheum.* **40**, 217-25. (1997).

Takayanagi, H., Oda H, Yamamoto S, Kawaguchi H, Tanaka S, Nishikawa T, Koshihara Y. A new mechanism of bone destruction in rheumatoid arthritis: synovial fibroblasts induce osteoclastogenesis. *Biochem Biophys Res Commun* **240**, 279-86 (1997).

Takayanagi, H., Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, Koshihara Y, Oda H, Nakamura K, Tanaka S. Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum* **43**, 259-69 (2000).

Thakker-Varia, S., Tozzi CA, Poiani GJ, Babiarz JP, Tatem L, Wilson FJ, Riley DJ. Expression of matrix-degrading enzymes in pulmonary vascular remodeling in the rat. *Am J Physiol* **275**, L398-406 (1998).

Thornton, R., Lane, P, Borghaei RC, Pease EA, Caro J, Mochan E. Interleukin 1 induces hypoxia-inducible factor 1 in human gingival and synovial fibroblasts. *Biochem J.* **350**, 307-12. (2000).

Tomita, T., Takeuchi E, Tomita N, Morishita R, Kaneko M, Yamamoto K, Nakase T, Seki H, Kato K, Kaneda Y, Ochi T. Suppressed severity of collagen-induced arthritis by in vivo transfection of nuclear factor kappaB decoy oligodeoxynucleotides as a gene therapy. *Arthritis Rheum.* **42**, 2532-42 (1999).

Tozzi, C., Thakker-Varia, S, Yu SY, Bennett RF, Peng BW, Poiani GJ, Wilson FJ, Riley DJ. Mast cell collagenase correlates with regression of pulmonary vascular remodeling in the rat. *Am J Respir Cell Mol Biol.* **18**, 497-510. (1998).

Trabandt, A., Aicher, WK, Gay RE, Sukhatme VP, Nilson-Hamilton M, Hamilton RT, McGhee JR, Fassbender HG, Gay S. Expression of the collagenolytic and Ras-induced cysteine proteinase cathepsin L and proliferation-associated oncogenes in synovial cells of MRL/l mice and patients with rheumatoid arthritis. *Matrix.* **10** (1990).

Trabandt, A., Gay, RE, Fassbender HG, Gay S. Cathepsin B in synovial cells at the site of joint destruction in rheumatoid arthritis. *Arthritis Rheum.* **34**, 1444-51. (1991).

Trabandt, A., Gay, RE, Gay S. Oncogene activation in rheumatoid synovium. *APMIS* **100**, 861-75. (1992a).

Trabandt, A., Aicher, WK, Gay RE, Sukhatme VP, Fassbender H-G, Gay S. Spontaneous expression of immediately-early response gene c-fos and egr-1 in collagenase-producing rheumatoid synovial fibroblasts. *Rheumatology International*. **12**, 53-59. (1992b).

Trang, L., Granstrom, E, Lovgren O. Levels of prostaglandins F2 alpha and E2 and thromboxane B2 in joint fluid in rheumatoid arthritis. *Scand J Rheumatol*. **6**, 151-4. (1977).

Trentham, D., Townes AS, Kang AH. Autoimmunity to type II collagen an experimental model of arthritis. *J Exp Med*. **146**, 857-68 (1977).

Treuhaff, P., McCarty, DJ. Synovial fluid pH, lactate, oxygen and carbon dioxide partial pressures in various joint diseases. *Arthritis and Rheumatism* **14**, 475-484 (1971).

Tsuji, M., Hirakawa K, Kato A, Fruji K. The possible role of c-fos expression in rheumatoid cartilage destruction. *J Rheumatol*. **27**, 1606-21 (2000).

Tsao, P., Suzuki T, Totsuka R, Murata T, Takagi T, Ohmachi Y, Fujimura H, Takata I. The effect of dexamethasone on the expression of activated NF-kappa B in adjuvant arthritis. *Clin Immunol Immunopathol* **82**, 173-8 (1997).

Turto, H., Lindy S, Uitto VJ, Wegelius O, Uitto J. Human leukocyte collagenase: characterization of enzyme kinetics by a new method. *Anal Biochem* **83**, 557-69 (1977).

Uesugi, M., Yoshida, K, Jasin HE. Inflammatory properties of IgG modified by oxygen radicals and peroxynitrite. *J Immunol*. **165**, 6532-7. (2000).

Urtasun, R. C., Chapman, J.D., Raleigh, J.A., Franko, A.J, Koch, C.J. Binding of 3H-misonidazole to solid human tumours as a measure of tumour hypoxia. *Int. J. Radiat. Oncol. Biol. Phys* **12**, 1263-1267 (1986).

van Eden, W., Holoshitz J, Nevo Z, Frenkel A, Klajman A, Cohen IR. Arthritis induced by a T-lymphocyte clone that responds to Mycobacterium tuberculosis and to cartilage proteoglycans. *Proc Natl Acad Sci U S A* **82**, 5117-20 (1985).

van Otteren, G., Standiford TJ, Kunkel SL, Danforth JM, Strieter RM. Alterations of ambient oxygen tension modulate the expression of tumor necrosis

factor and macrophage inflammatory protein-1 alpha from murine alveolar macrophages. *Am J Respir Cell Mol Biol* **13**, 399-409 (1995).

van Tienhoven, E., van Kooten PJ, Veenstra JG, van der Hage MH, van Eden W, Broeren CP. Induction of experimental autoimmune arthritis by a public epitope of the T cell receptor variable alpha domain of an arthritogenic T cell clone. *Eur J Immunol* **30**, 2164-71 (2000).

Vandenbunder, B., Pardanaud L, Jaffredo T, Mirabel MA, Stehelin D. Complementary patterns of expression of c-ets 1, c-myb and c-myc in the blood-forming system of the chick embryo. *Development*. **107**, 265-74 (1989).

Vandenbunder, B., Queva C, Desbiens X, Wernert N, Stehelin D. Expression of the transcription factor c-Ets1 correlates with the occurrence of invasive processes during normal and pathological development. *Invasion Metastasis*. **14**, 198-209 (1994).

Vane, J. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol*. **231**, 232-5 (1971).

van't Hof, R., Hocking L, Wright PK, Ralston SH. Nitric oxide is a mediator of apoptosis in the rheumatoid joint. *Rheumatology (Oxford)*. **39**, 1004-8 (2000).

Venables, P., Pawlowski T, Mumford PA, Brown C, Crawford DH, Maini RN. Reaction of antibodies to rheumatoid arthritis nuclear antigen with a synthetic peptide corresponding to part of Epstein-Barr nuclear antigen 1. *Ann Rheum Dis* **47**, 270-9 (1988).

Vincenti, M., Clark IM, Brinckerhoff CE. Using inhibitors of metalloproteinases to treat arthritis. Easier said than done? *Arthritis and Rheumatism* **37**, 1115-26 (1994).

Vincenti, M., Brinckerhoff CE. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. *Get Arthritis Res* **4**, 157-64 (2002).

Vingsbo, C., Jonsson R, Holmdahl R. Avridine-induced arthritis in rats; a T cell-dependent chronic disease influenced both by MHC genes and by non-MHC genes. *Clin Exp Immunol*. **99**, 359-63 (1995).

Volin, M., Woods, JM, Amin MA, Connors MA, Harlow LA, Koch AE. Fractalkine: a novel angiogenic chemokine in rheumatoid arthritis. *Am J Pathol.* **159**, 1521-30. (2001).

Wallis, W., Simkin, PA, Nelp WB. Low synovial clearance of iodide provides evidence of hypoperfusion in chronic rheumatoid synovitis. *Arthritis Rheum.* **28**, 1096-1104. (1985a).

Wallis, W., Simkin PA, Nelp WB, Foster DM. Intraarticular volume and clearance in human synovial effusions. *Arthritis and Rheumatism* **28**, 441-9 (1985b).

Walsh, D., Wade M, Mapp PI, Blake DR. Focally regulated endothelial proliferation and cell death in human synovium. *J Pathol* **152**, 691-702 (1998).

Walsh, D. Angiogenesis and arthritis. *Rheumatology (Oxford)*. **38**, 103-12 (1999).

Weinblatt, M., Kremer JM, Bankhurst AD, Bulpitt KJ, Fleischmann RM, Fox RI, Jackson CG, Lange M, Burge DJ. A trial of etanercept, a recombinant tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. *N Engl J Med.* **340**, 253-9 (1999).

Wenger, R., Gassman, M. Oxygen(es) and the hypoxia-inducible factor-1. *Biol Chem* **378**, 609-16 (1997).

Wernert, N., Raes, MB, Lassalle P, Dehouck MP, Gosselin B, Vandenbunder B, Stehelin D. C-ets1 proto-oncogene is a transcription factor expressed in endothelial cells during tumor vascularization and other forms of angiogenesis in humans. *Am J Pathol.* **140**, 119-27. (1992).

Wernert, N., Gilles F, Fafeur V, Bouali F, Raes MB, Pyke C, Dupressoir T, Seitz G, Vandenbunder B, Stehelin D. Stromal expression of c-Ets1 transcription factor correlates with tumor invasion. *Cancer Res.* **54**, 5683-8 (1994).

Wernert, N., Justen HP, Rothe M, Behrens P, Dreschers S, Neuhaus T, Florin A, Sachinidis A, Vetter H, Ko Y. The Ets 1 transcription factor is upregulated during inflammatory angiogenesis in rheumatoid arthritis. *J Mol Med.* **80**, 258-66 (2002).

White, K., Marletta MA. Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry*. **31**, 6627-31 (1992).

Wiesener, M., Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, et al. Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood* **92**, 2260-8 (1998).

Williams, R., Feldmann M, Maini RN. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc Natl Acad Sci U S A*. **89**, 9784-8 (1992).

Willis, D. Expression and modulatory effects of heme oxygenase in acute inflammation in the rat. *Inflamm Res. Suppl.* **2**. S218-20.

Willkens, R., Watson MA. Methotrexate: a perspective of its use in the treatment of rheumatic diseases. *J Lab Clin Med*. **100**, 314-21 (1982).

Winrow, V. in *Inducible Enzymes in the Inflammatory Response* (ed. Willoughby, D., Tomlinson A) (Birkhauser Verlag, 1999).

Winyard, P., Tatzber, F, Esterbauer H, Kus ML, Blake DR, Morris CJ. Presence of foam cells containing oxidised low density lipoprotein in the synovial membrane from patients with rheumatoid arthritis. *Ann Rheum Dis* **52**, 677-80. (1993).

Wright, T., Hoffman, RD, Nishijima J, Jakoi L, Snyderman R, Shin HS. Leukocyte chemoattraction by 1,2-diacylglycerol. *Proc Natl Acad Sci U S A*. **85**, 1869-73 (1988).

Wu , K. Inducible cyclooxygenase and nitric oxide synthase. *Adv Pharmacol* **1995**, 179-207 (1995).

Xie, Q., Nathan C. The high-output nitric oxide pathway: role and regulation. *J Leukoc Biol* **56**, 576-82 (1994).

Xue, C., Takahashi, M, Hasunuma T, Aono H, Yamamoto K, Yoshino S, Sumida T and Nishioka K. Characterisation of fibroblast-like cells in pannus lesions of patients with rheumatoid arthritis sharing properties of fibroblasts and chondrocytes. *Annals of Rheumatic Disease* **56**, 262-267 (1997).

Yanni, G., Whelan A, Feighery C, Fitzgerald O, Bresnihan B. Morphometric analysis of synovial membrane blood vessels in rheumatoid arthritis: associations

with the immunohistologic features, synovial fluid cytokine levels and the clinical course. *J Rheumatol.* **20**, 634-8 (1993).

Yoshino, S., Schlipkoter E, Kinne R, Hunig T, Emmrich F. Suppression and prevention of adjuvant arthritis in rats by a monoclonal antibody to the alpha/beta T cell receptor. *Eur J Immunol.* **20**, 2805-8 (1990).

Zhang, J., Behrooz, A, Ismail-Beigi F. Regulation of glucose transport by hypoxia. *Am J Kidney Dis.* **34**, 189-202. (1999).

Zhang, Z., Naughton, D, Winyard PG, Benjamin N, Blake DR, Symons MC. Generation of nitric oxide by a nitrite reductase activity of xanthine oxidase: a potential pathway for nitric oxide formation in the absence of nitric oxide synthase activity. *Biochem Biophys Res Commun.* **249**, 767-72 (1998a).

Zhang, Z., Blake, DR, Stevens CR, Kanczler JM, Winyard PG, Symons MC, Benboubetra M, Harrison R. A reappraisal of xanthine dehydrogenase and oxidase in hypoxic reperfusion injury: the role of NADH as an electron donor. *Free Radic Res.* **28**, 151-64. (1998b).

Zvaifler, N., Firestein, GS. Pannus and pannocytes. Alternative models of joint destruction in rheumatoid arthritis. *Arthritis and Rheumatism* **37**, 783-789 (1994).

Zvaifler, N., Tsai, V, Alsalameh S, von Kempis J, Firestein GS and Lotz M. Pannocytes: Distinctive cells found in rheumatoid arthritis articular cartilage erosions. *American Journal of Pathology* **150**, 1125-1138. (1997).

Appendix A

Stacking Buffer - 100ml

Tris base: 6.05g

ddWater: 100ml

pH 6.8 with HCl

Running Buffer - 100ml

Tris base: 18.17g

ddWater: 100ml

pH8.8 with HCl

7.5% Acrylamide Running Gel

Running buffer: 6.5ml

ddWater: 13.7ml

10% SDS: 0.25ml

40% Acrylamide: 4.64ml

TEMED: 10 μ l

APS: 100 μ l

5% Acrylamide Stacking Gel

Stacking buffer: 0.5ml

ddWater: 3.036ml

1 0% SDS: 40 μ l

40% Acrylamide: 0.38ml

TEMED: 4 μ l

APS: 40 μ l

Reducing Buffer

ddWater: 6.75ml

Glycerol: 1.6ml

SDS: 0.2g

Bromophenol blue: 1mg

Take 750 μ l of above solution and add 2-mercaptoethanol: 50 μ l

Electrophoresis Buffer

Glycine: 14.4g

Tris base: 3g

10% SDS: 10ml

Make up to 1000ml with dd water

Blotting Buffer

Glycine: 14.4g

Tris base: 3g

10% SDS: 10ml

Methanol: 200ml

Make up to 1000ml with dd water

Coomassie Blue Staining Solution

Coomassie Brilliant Blue: 1.25mg

Methanol: 45ml

6M Acetic Acid: 10ml (34ml: glacial acetic acid + 63ml dd water = 6M solution)

Make up to 100ml with dd water.

Appendix B

The Transcription Factors Hypoxia-Inducible Factor 1 α and Ets-1 Colocalize in the Hypoxic Synovium of Inflamed Joints in Adjuvant-Induced Arthritis

C. L. Peters,¹ C. J. Morris,¹ P. I. Mapp,¹ D. R. Blake,¹ C. E. Lewis,² and V. R. Winrow¹

Objective. To determine the relationship between hypoxia and the expression of Ets-1 and hypoxia-inducible factor 1 α (HIF-1 α) in both normal and inflamed joints. Adjuvant-induced arthritis (AIA) was used as the model system, since it mirrors many aspects of the pathology of rheumatoid arthritis.

Methods. Adjuvant arthritis was induced in a group of 10 female Lewis rats. A second group of 10 uninjected female Lewis rats served as naive controls. When a maximum clinical joint score was achieved in the AIA group, all 20 rats were injected with the specific hypoxic cell marker Hypoxyprobe-1 and subsequently killed. Hypoxyprobe-1 adducts, Ets-1, and HIF-1 α were localized in the joints of the hind feet from these groups using immunohistochemistry.

Results. Compared with the joints from control rats, inflamed joints contained markedly more cells with Hypoxyprobe-1 adduct immunoreactivity, Ets-1-immunoreactive nuclei, and nuclear immunoreactivity for both Ets-1 and HIF-1 α .

Conclusion. Our results demonstrate the presence of hypoxia in inflamed joints in this experimental model of arthritis. The colocalization of Ets-1 and HIF-1 α in these hypoxic areas suggests that hypoxia

may induce Ets-1 and HIF-1 α expression during joint inflammation.

Rheumatoid arthritis (RA) is characterized by a progressive and proliferative synovitis that is invasive but not malignant, which results in joint destruction. The synovium is hypoxic, probably as a result of synovial tissue proliferation outpacing angiogenesis (1). Hypoxia is a potent inducer of cytokines, matrix-degrading enzymes, and angiogenic factors and plays a central role in the normal inflammatory response. Moreover, hypoxia-driven angiogenesis is critical for extracellular matrix formation and tissue remodeling (2). The Ets-1 transcription factor, which was identified in endothelial cells during angiogenesis and as a marker of tumor invasion (3), can be induced following activation of a second transcription factor, hypoxia-inducible factor 1 (HIF-1), and both Ets-1 and HIF-1 are overexpressed in rheumatoid synovial tissue (4,5).

Since inhibitors of angiogenesis can modulate disease in experimental arthritis (6), we examined the relationship between cellular hypoxia, HIF-1, and Ets-1 expression in the joints of rats with adjuvant-induced arthritis (AIA). This model mirrors much of the pathology of RA, including synovial tissue proliferation, pannus formation, and the destruction of bone and cartilage in the synovial joint. To visualize hypoxic cells, we used Hypoxyprobe-1 (pimonidazole; Chemicon Europe, Chesham, UK), a recently developed compound which is taken up by all body tissues in vivo but which binds only to cells with a low oxygen tension ($P_{O_2} \leq 10$ mm Hg at 37°C). The monoclonal antibody Hypoxyprobe-1-Mab1 binds to protein adducts of Hypoxyprobe produced in hypoxic cells, allowing their immunohistochemical identification in tissues ex vivo. The reductive metabolism of this hypoxia marker is highly specifically regulated by oxygen tension indepen-

Ms Peters, Dr. Morris, and Dr. Winrow's work was supported by grants B0583, C0670, and M561 from the Arthritis Research Campaign (UK). Dr. Lewis's work was supported by grants from the Medical Research Council of Great Britain, the Biotechnology and Biological Sciences Research Council of the UK, the Engineering and Physical Sciences Research Council of the UK, and the Breast Cancer Campaign.

¹C. L. Peters, BSc, C. J. Morris, PhD, P. I. Mapp, PhD, D. R. Blake, FRCP, V. R. Winrow, PhD: University of Bath, Bath, UK; ²C. E. Lewis, DPhil: University of Sheffield Medical School, Sheffield, UK.

Address correspondence and reprint requests to C. J. Morris, PhD, Department of Medical Sciences, University of Bath, Claverton Down, Bath BA2 7AY, UK. E-mail: mpsejm@bath.ac.uk.

Submitted for publication January 31, 2003; accepted in revised form September 25, 2003.

dently of the pyridine nucleotide redox state (7). Pimonidazole was first used to quantify hypoxic tumor tissue as an index of radiation resistance (8) and has since become widely used in cancer research.

MATERIALS AND METHODS

Induction of adjuvant arthritis. Female Lewis rats (Charles River UK, Kent, UK) weighing 130–150 grams were housed in 4 groups of 5 rats and were exposed to a 12-hour light/dark cycle and a mean temperature of 20°C ($\pm 2^\circ\text{C}$). All animals were allowed access to tap water and a standard rat diet (SDS, Witham, UK) ad libitum. Adjuvant arthritis was induced using a method we have previously described (9). AIA rats ($n = 10$) were injected intradermally into the base of the tail with 100 μl of a suspension of pulverized, heat-killed *Mycobacterium tuberculosis* (Central Veterinary Laboratory, Weybridge, UK), 10 mg/ml in light paraffin oil. A second group of uninjected rats ($n = 10$) served as naive controls.

Disease progression was monitored daily using a standard clinical scoring system (0 = no inflammation, 4 = severe joint inflammation and deformity) (9). When a maximum clinical score of 4 was reached in 1 hind foot, the animals were killed by cervical dislocation. Naive rats were killed at the same time. Six hours prior to death, all 20 rats were injected intraperitoneally with 60 mg/kg of Hypoxyprobe-1 (Chemicon Europe) dissolved in 1 ml of 0.8% sterile saline (Sigma, Poole, UK). Optimization studies had been carried out previously (data not shown) to determine how tissue hypoxia detected by Hypoxyprobe adducts varied with AIA disease progression. Hypoxia was detectable as soon as clinical symptoms developed and increased with the degree of inflammation, as quantified by the joint score. The end point of this study was the maximum joint score, as dictated by The Home Office (UK).

Tissue sectioning/mounting. Five micrometer-thick alternate downward- and upward-facing (flip-flop) and consecutive sections were cut from decalcified hind feet (joint score 4). The sections were mounted on glass slides and dried in an oven at 60°C overnight.

Immunostaining for Hypoxyprobe. Pronase (0.01%)–treated dewaxed sections were blocked with normal horse serum in phosphate buffered saline (PBS) (1/200 volume/volume) for 20 minutes at ambient temperature. Sections were overlaid with 200 μl of mouse monoclonal anti-Hypoxyprobe antibody (Hypoxyprobe-1-Mab1; Chemicon Europe), diluted 1:50 in PBS containing 1/200 v/v normal horse serum, and kept at 4°C overnight. Negative controls were incubated in the same dilution of an isotype-matched mouse monoclonal antibody to an irrelevant antigen, *Aspergillus niger* glucose oxidase. Bound antibody was detected using an avidin-biotin-peroxidase complex (ABC) phosphatase kit (Vector Laboratories, Peterborough, UK), and phosphatase activity was revealed using a fast red substrate. All slides were counterstained with Mayer's hematoxylin, blued in tap water, and mounted in Aquamount under standard conditions.

Immunostaining for Ets-1. Dewaxed tissue sections were blocked with normal goat serum in PBS (1/200 v/v) for 20 minutes at ambient temperature. Rabbit polyclonal anti-Ets-1

antibody (Autogen Bioclear, Calne, UK) in PBS (1/500 v/v) was applied to the slides, which were kept at 4°C overnight. Negative controls were similarly treated in the same dilution of normal rabbit IgG1. Bound antibody was detected using an ABC phosphatase kit, and phosphatase activity was revealed using a fast red substrate. All tissue sections were counterstained with Mayer's hematoxylin.

Immunostaining for HIF-1 α . Dewaxed tissue sections were stained for HIF-1 α using a catalyzed signal amplification system. Sections were washed in 3% H_2O_2 in distilled water followed by PBS and blocked with protein block for 20 minutes at ambient temperature. A mouse anti-HIF-1 α antibody (Abcam, Cambridge, UK) diluted 1:500 in background-reducing antibody diluent (DakoCytomation, Ely, UK) was applied to the sections, which were maintained at 4°C overnight. Negative controls were similarly maintained in the same dilution of an isotype-matched mouse monoclonal antibody to an irrelevant antigen, *A. niger* glucose oxidase. Sections were treated with link antibody followed by streptavidin-biotin complex, amplification agent, and, finally, streptavidin-peroxidase reagent. Peroxidase activity was revealed using a 3,3'-diaminobenzidine substrate, and sections were counterstained with Mayer's hematoxylin.

Image analysis. Sections were examined by two independent observers using an Axioskop II microscope (Zeiss, Wetzlar, Germany) fitted with a 3-charge-coupled device camera (JVC, Tokyo, Japan) for digital imaging and white light illumination. Photographic records were stored as jpeg images.

Quantitative analysis of hypoxia by detection of Hypoxyprobe-1 adducts. We used a modified version of a method used for the quantification of Hypoxyprobe-1 adducts in mouse radiation-induced fibrosarcoma-1 tumors (10). Three sections from each of 10 hind feet were assessed using a 20 \times microscope objective. For arthritic animals, only hind feet with a maximum clinical score of 4 ($n = 10$) were used. Equivalent hind feet of naive animals ($n = 10$) were also quantified. Image analysis was carried out on all areas of joint tissue to determine the percentage of stained area compared with unstained area.

Sections were assessed in a blinded manner using a program written for the KS 300 Imaging System, version 3.0 (Imaging Associates, Bicester, UK). This program analyzes a framed area of 800 \times 600 pixels. It removes from the analysis any area $< 10 \mu\text{m}$, thus removing small histologic artifacts. Large histologic artifacts were manually removed from the analysis by outlining them. Hypoxic regions were thresholded based upon a pimonidazole adduct density converted to a red-blue-green level which was consistent throughout the images. The spatial transition between peak density and 0% (nonhypoxic area) was distinct, providing a reproducible method to demarcate hypoxic areas. Finally, the program calculated the demarcated area as a percentage of the whole framed area. These data were then tabulated using Prism software (GraphPad Software, San Diego, CA) for statistical analysis.

Quantitative analysis of positive HIF-1 α and Ets-1 immunoreactivity. Three alternate downward-facing and upward-facing (flip-flop) sections from 10 hind feet were surveyed for areas of identical tissue architecture using a 40 \times microscope objective. Image analysis was limited to synovial tissue lining the joint cavity and synovial tissue attached to the bone. A program written for the KS 300 Imaging System

version 3.0 was used. This program enabled the counting of nuclei with positive immunoreactivity for Ets-1 to be expressed as a proportion of the total cell population counted in a framed area of $100 \times 100 \mu\text{m}$. The proportion of nuclei showing immunoreactivity for both Ets-1 and HIF-1 α was also assessed. These data were then tabulated using Prism software (Graph-Pad Software).

RESULTS

Clinically detectable swelling was present in the distal joints of the hind feet from day 11; this increased in severity and led to ankle joint deformation by day 15. At this time, sections of AIA rat feet showed synovial hyperplasia, increased cellular infiltration, and bone destruction.

Quantitative analysis. In hind feet from AIA rats, there was a highly significant increase ($P < 0.0001$) in the proportion of cells having nuclei with positive immunoreactivity for Ets-1 compared with hind feet from naive control rats (Figures 1A, 2A, and 2B). AIA rats also had significantly ($P < 0.0001$) more hypoxic tissue compared with naive control rats, as demonstrated by positive immunoreactivity for Hypoxyprobe-1 adducts, measured by the binding of Hypoxyprobe-1-Mab1 (Figures 1B, 2C, and 2D). Hind feet from AIA rats showed a highly significant increase ($P < 0.0001$) in the proportion of cells having nuclei with positive immunoreactivity for both Ets-1 and HIF-1 α compared with hind feet from naive control rats (Figures 1C, 2E, and 2F). Statistical comparisons were made using the Mann-Whitney U test.

Immunohistochemical assessment. In the foot tissue of AIA rats, positive immunoreactivity for both Hypoxyprobe-1 adducts and HIF-1 α involved all structures, including the synovium, pannus, bone marrow, and articular cartilage chondrocytes. Immunostained sections showed that positive immunoreactivity for Ets-1 was mainly found in the inflammatory infiltrate and invasive pannus of the inflamed synovial joint (Figure 2A). The histologically normal hind feet from naive rats showed a low level of positive immunoreactivity for Ets-1 in the subintimal synoviocytes (Figure 2B). The same was true of positive immunoreactivity for Hypoxyprobe-1 adducts (Figures 2C and D). In cell nuclei of AIA rats, positive immunoreactivity for Ets-1 (Figure 2E) colocalized with strong positive immunoreactivity for HIF-1 α in 5- μm flip-flop sections (Figure 2F), mainly in inflammatory infiltrate and invasive pannus of the inflamed synovial joint. Interestingly, colocalization was not 100% (Figures 1C, 2E, and 2F). Figures 2G and H represent nonstaining negative controls

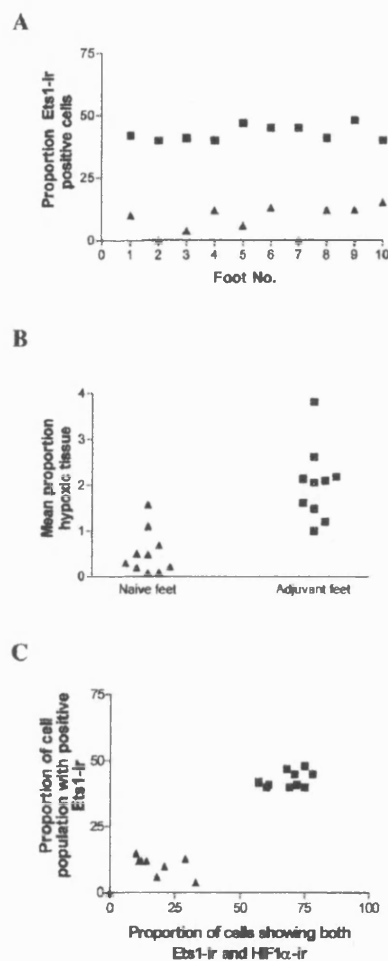


Figure 1. A, Proportion of cells expressing Ets-1 transcription factor in single hind feet ($n = 10$). Ets-1 expression in the foot joints of rats with adjuvant-induced arthritis (AIA) (■) is significantly higher than that in the foot joints of naive rats (▲) ($P < 0.0001$ by Mann-Whitney U test). B, Percentage of total area examined that was hypoxic ($\text{PO}_2 \leq 10 \text{ mm Hg}$), as indicated by positive immunoreactivity for Hypoxyprobe-1 adducts. The percentage of hypoxic tissue in the hind feet of AIA rats is significantly higher than that in the hind feet of naive rats ($P < 0.0001$ by Mann-Whitney U test). C, Proportion of the cell population coexpressing Ets-1 transcription factor and hypoxia-inducible factor 1 α (HIF-1 α) in each of 10 hind foot samples. The hind feet from AIA rats showed a highly significant increase in the proportion of cells having nuclei with positive immunoreactivity for both Ets-1 and HIF-1 α compared with the hind feet from naive rats ($P < 0.0001$ by Mann-Whitney U test). Ets1-ir = immunoreactivity for Ets-1; HIF1 α -ir = immunoreactivity for HIF-1 α .

(isotype-matched mouse monoclonal antibody to an irrelevant antigen [*A niger* glucose oxidase] and rabbit IgG1, respectively).

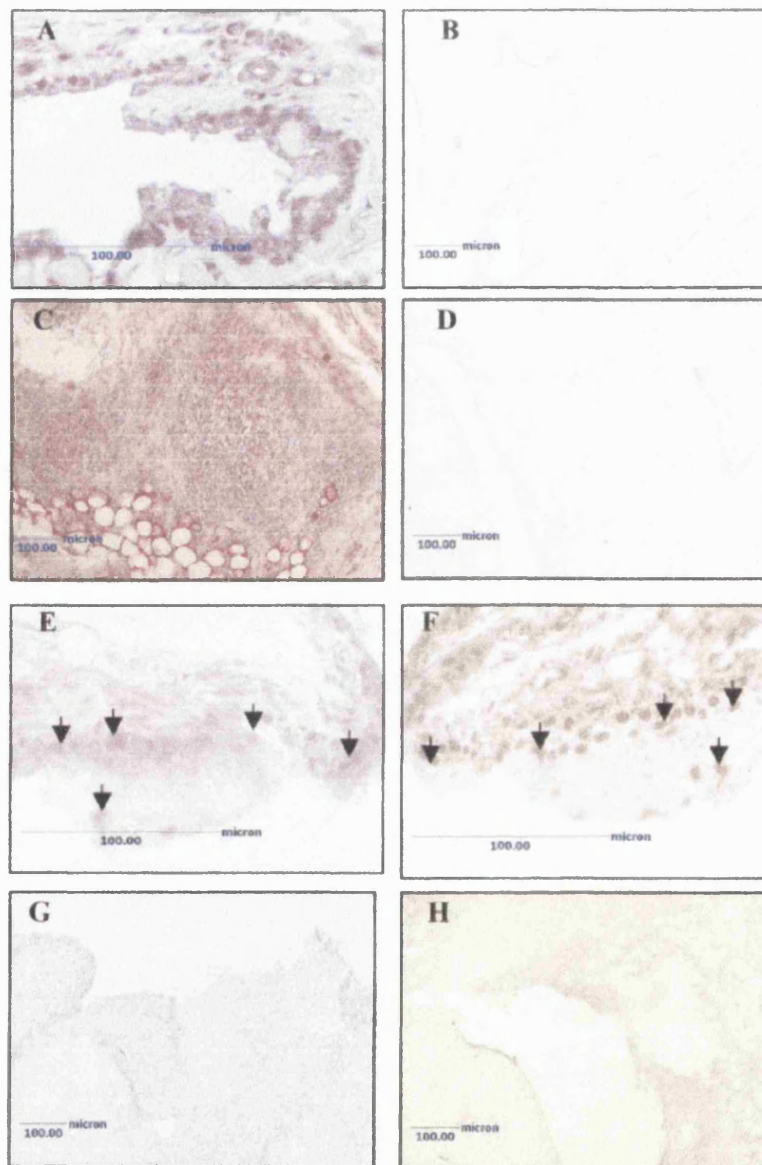


Figure 2. Immunohistochemical assessments of immunoreactivity in hind foot sections from AIA and naive rats. **A**, Hind foot from an AIA rat showing nuclear localization of positive immunoreactivity (red) for Ets-1. **B**, Hind foot from a naive rat showing a low level of positive immunoreactivity for Ets-1. **C**, Hind foot from an AIA rat showing inflammatory infiltrate with positive immunoreactivity (red) for Hypoxyprome-1 adducts. **D**, In the hind foot from a naive rat, the synovium shows no immunoreactivity for Hypoxyprome-1 adducts. **E**, In the hind foot from an AIA rat, the synovium shows proliferating synoviocytes with positive immunoreactivity (red) for Ets-1 (**arrows**). **F**, Alternate flip-flop section of same hind foot shown in **E** reveals nuclei with strong positive immunoreactivity for HIF-1 α (brown; **arrows**) colocalizing with positive immunoreactivity for Ets-1. Colocalization does not involve all nuclei (see Figure 1C). **G**, Isotype-matched mouse monoclonal antibody to an irrelevant antigen (*Aspergillus niger* glucose oxidase) as a negative control for mouse antibodies in inflamed tissue. **H**, Rabbit IgG1 as a negative control in inflamed tissue. See Figure 1 for definitions.

DISCUSSION

Using a definitive marker, we have shown that joint inflammation is associated with an increase in cellular hypoxia. This is concordant with the situation in the rheumatoid joint as demonstrated by HIF-1 α expression (11). Since the Ets-1 promoter has been shown to contain hypoxia-responsive element (HRE)-like sequences (12), we would expect increased cellular hypoxia to be associated with a concurrent increase in the expression of Ets-1. This was verified by our results: the joints of all 10 feet from the AIA rats had a minimum of 40% Ets-1-positive nuclei in the total cell population, while in the 10 naive joint samples, no more than 15% of the nuclei expressed detectable Ets-1. Furthermore, 65–75% of nuclei expressing Ets-1 in the samples from AIA rats also expressed HIF-1 α . This clearly demonstrates an induction of hypoxia and expression of Ets-1 during the inflammatory response in AIA rat joints.

Other investigators have demonstrated that Ets-1 transcription factor up-regulation correlates with tumor invasion (3), and our localization of Ets-1 in the invasive pannus is indicative of its involvement in the invasion of synovial joint cartilage and bone by proliferating synovium. Additionally, our demonstration that Ets-1 and HIF-1 α colocalize in areas of hypoxia in inflamed synovial tissue suggests that both hypoxia and HIF-1 α may be involved in the up-regulation of Ets-1 during joint inflammation.

The broad range of target genes for Ets-1 has been extensively reviewed (13). Of particular relevance to the pathology of arthritis is its involvement in angiogenesis and extracellular matrix remodeling. Matrix metalloproteinases (MMPs), such as collagenase 1 (MMP-1), stromelysin 1 (MMP-3), and gelatinase B (MMP-9), and tissue inhibitors of metalloproteinases (TIMPs), such as TIMP-1, are all gene targets of Ets-1 and have been shown to be elevated in patients with RA (14).

Increased hypoxia has been associated with RA (2), and this has led to the concept of hypoxia-targeted therapies, including gene therapy. HIF-1 α expression is abundant in rheumatoid synovium (5), so strategies that target HIF-1 could be effective in treating RA. Hypoxia can also be used to target gene therapy to certain diseased areas. This concept exploits the fact that HIF-1 is degraded by exposure to oxygen but activated and stabilized under conditions of hypoxia (15). The expression of a therapeutic gene of interest is regulated by the inclusion of an HRE upstream of the therapeutic gene in an expression vector. At this time, we cannot confirm that Ets-1 has a role in hypoxia-regulated gene therapy

for arthritis, since it is not fully understood whether other factors present *in vivo* would also activate Ets-1 in a gene transfer delivery vehicle. A previous report from investigators in our group (5) suggested that macrophages are the main expressors of HIF-1 α during the inflammatory response in RA. However, our study reported here indicates that other cells appear to be involved as well.

Further research is under way to identify the cell phenotype expressing both HIF-1 α and Ets-1. Nevertheless, because we have demonstrated both increased hypoxia and Ets-1 expression in cells at the site of cartilage and bone destruction, these could be targeted using HIF-1-mediated systems to limit joint damage. Our study clearly provides further evidence that AIA is a suitable model for *in vivo* research into the likely *in vivo* efficacy of hypoxia-regulated therapies in the treatment of RA.

ACKNOWLEDGMENTS

We thank Drs. P. Lewis and C. Chatfield, statisticians at the University of Bath, for their valuable advice.

REFERENCES

1. Stevens CR, Blake DR, Merry P, Revell PA, Levick JR. A comparative study by morphometry of the microvasculature in normal and rheumatoid synovium. *Arthritis Rheum* 1991;34:1508–13.
2. Steinbrech DS, Longaker MT, Mehrara BJ, Saadeh PB, Chin GS, Gerrets RP, et al. Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation. *J Surg Res* 1999;84:127–33.
3. Wernert N, Gilles F, Fafeur V, Bouali F, Raes MB, Pyke C. Stromal expression of c-Ets1 transcription factor correlates with tumor invasion. *Cancer Res* 1994;54:5683–8.
4. Wernert N, Justen HP, Rothe M, Behrens P, Dreschers S, Neuhaus T, et al. The Ets 1 transcription factor is upregulated during inflammatory angiogenesis in rheumatoid arthritis. *J Mol Med* 2002;80:258–66.
5. Hollander AP, Corke KP, Freemont AJ, Lewis CE. Expression of hypoxia-inducible factor 1 α by macrophages in the rheumatoid synovium: implications for targeting of therapeutic genes to the inflamed joint. *Arthritis Rheum* 2001;44:1540–4.
6. Lutun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, et al. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 2002;8:831–40.
7. Arteel GE, Thurman RG, Raleigh JA. Reductive metabolism of the hypoxia marker pimonidazole is regulated by oxygen tension independent of the pyridine nucleotide redox state. *Eur J Biochem* 1998;253:743–50.
8. Kennedy AS, Raleigh JA, Perez GM, Calkins DP, Thrall DE, Novotny DB, et al. Proliferation and hypoxia in human squamous

- cell carcinoma of the cervix: first report of combined immunohistochemical assays. *Int J Radiat Oncol Biol Phys* 1997;37:897-905.
9. Andrews FJ, Morris CJ, Kondratowicz G, Blake DR. Effect of iron chelation on inflammatory joint disease. *Ann Rheum Dis* 1987;46:327-33.
 10. Pogue BW, Paulsen KD, O'Hara JA, Wilmot CM, Swartz HM. Estimation of oxygen distribution in RIF-1 tumors by diffusion model-based interpretation of pimonidazole hypoxia and Eppendorf measurements. *Radiat Res* 1995;155:15-25.
 11. Hitchon C, Wong K, Ma G, Reed J, Lytle D, El-Gabalawy H. Hypoxia-induced production of stromal cell-derived factor 1 (CXCL12) and vascular endothelial growth factor by synovial fibroblasts. *Arthritis Rheum* 2002;46:2587-97.
 12. Oikawa M, Abe M, Kurosawa H, Hida W, Shirato K, Sato Y. Hypoxia induces transcription factor ETS-1 via the activity of hypoxia-inducible factor-1. *Biochem Biophys Res Commun* 2001;289:39-43.
 13. Lelievre E, Lionneton F, Soncin F, Vandenbunder B. The Ets family contains transcriptional activators and repressors involved in angiogenesis. *Int J Biochem Cell Biol* 2001;4:391-407.
 14. Klimiuk PA, Sierakowski S, Latosiewicz R, Cylwik B, Skowronski J, Chwiecko J. Serum matrix metalloproteinases and tissue inhibitors of metalloproteinases in different histological variants of rheumatoid synovitis. *Rheumatology (Oxford)* 2002;41:78-87.
 15. Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, et al. Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood* 1998;92:2260-8.

Abstracts

Peters CL, PI Mapp, VR Winrow, CE Lewis, DR Blake, CJ Morris
Does Hypoxia Promote Proliferative Synovitis?
Inflammation Research Vol.52. Supplement2. pp.S103. (2003).

Peters CL, PI Mapp, DR Blake, CJ Morris.
Effect of Hypoxia on Transcription Factor Ets-1 in the inflamed joint. Arthritis and Rheumatism. Vol. 46. Number9 (Supplement) pp. S546. (2002).